EXPERIMENTAL CELL RESEARCH

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THE INTERNATIONAL SOCIETY FOR CELL BIOLOGY

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VOLUME 8 · APRIL 1955 · NUMBER 2

ACADEMIC PRESS INC., PUBLISHERS, NEW YORK, N.Y.

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Experimental Cell Research, **8**, *259–278 (1955)*

MICROSPECTROPHOTOMETRY OF CELL NUCLEUS STAINED BY FEULGEN REACTION

I. MICROSPECTROPHOTOMETRIC APPARATUS WITHOUT SCHWARZSCHILD-VILLIGER EFFECT

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Received April 28, 1954

By the technique of microspectrophotometry pioneered by Caspersson in 1936 (2), it has become possible to determine the minute quantity of light-absorbing substances distributed in an *individual* cell or a nucleus. The amount of these structural or functional substances is of the order of 10^{-12} gram in weight, or 100 cubic micron in volume, the measurement of which is far beyond the scope of the usual chemical analysis, by which we can determine only the *mean* content of these important substances among numerous cells or nuclei of the same species. The knowledge of the mean value is sometimes of no use in biology, as each individual cell of the same kind often shows variability with regard to the amount of its content, to the measurement of which the microspectrophotometric method is indispensable.

Unfortunately, biological conclusions about the same theme obtained through microspectrophotometry by various investigators are sometimes inconsistent with each other, the most remarkable example being the problem of the constancy (1, 4, 8, 17, 18) or the variability (3, 5, 10, 26, 27) of the content of desoxypentosenucleic acid (DNA) in individual diploid nucleus from the same species. A part of these discrepancies may be due to somewhat indefinite factors involved in the Feulgen reaction for staining nuclei (6, 7, 8), but the most part of them seems to be attributed to the careless use of microspectrophotometric equipments, the optical system of which is not free from the "Schwarzschild-Villiger effect (S–V effect)", which has been curiously ignored since the beginning of the microspectrophotometry.

In view of these, we constructed a microspectrophotometric apparatus in which the S–V effect is eliminated, and by using it we have obtained several noticeable results. In the series of these reports, one part will deal with the description of the apparatus, another with the measurement of the content

of DNA in nuclei by our apparatus, and a third part with the problem of the constancy or variability of the content of DNA in individual nucleus from the same species of cells.

SCHWARZSCHILD-VILLIGER EFFECT

In 1906, K. Schwarzschild and W. Villiger (20) found in microdensitometry of developed photographic plates, that serious errors are sometimes introduced in the measurement of the light transmittance of a minute portion of the plate, whenever the large outer area surrounding the portion in question is illuminated by the measuring light flux. The magnitude of the error often amounts to as high as 100–1000 per cent of the true value of the transmittance. The fact has been noticed especially by makers of precision microphotometers, and the term "Schwarzschild-Villiger effect" appears in Harrison's description (6) about densitometers and microphotometers.

The origin of the S-V effect is the flare light in the optical system of microphotometers which makes a magnified image of the portion of the photographic plate to be measured. The main part of the light flux from any point on the plate is sharply focused on the image plane, but a part of it is reflected back and forth indefinitely at every air-to-glass interface of the optical system, or scattered by minute bubbles in optical glasses or by small defects of polishing or contaminations at lens surfaces, and finally illuminates wide areas of the image plane around the sharply defined image of the point in question. This is the flare light that causes the S-V effect.

Thus when we measure the transmittance of a small portion of the photographic plate, densely blackened and surrounded by transparent background as in the case of the image of a star or spectral line, by using a simple microphotometer in which a large portion of the plate is illuminated by the measuring light flux, then the flare light originated from every brighter point in the surrounding portion on the plate is added, in the image plane, to the very image of the small portion in question, and the measured value of its transmittance is unduly and sometimes seriously enhanced.

The circumstances are quite same, or even worse in the case of microspectrophotometry, worse because the magnifying optical system in microspectrophotometric apparatus is usually more complicated than in microphotometers, i.e. it consists of much more lenses, while the amount of the flare light increases rapidly with the number of air-to-glass interfaces of the system. For example (13), a deeply stained isolated nucleus of rat liver with the true transmittance of 1.1 per cent for green light of λ 5461Å, shows

an apparent transmittance of 20.0 per cent (18 times the true value!) by illuminating the wide area of ca. 0.65 mm in diameter containing the nucleus, the number of air-to-glass interfaces being thirteen, namely 7 in an oil-immersed objective, 4 in a Huygenian eye-piece, and 2 in a reflection prism.

It should be remarked here that the term flare light in our description implies only those light fluxes which are caused by multiple internal reflections of light and by scattering of light in the optical parts, and not so-called mechanical flare, which is caused by unintentional reflections of light at inner walls of tubes, etc., and which can be removed by careful designing and use of mechanical parts of the optical system (7) (Fig. 1).

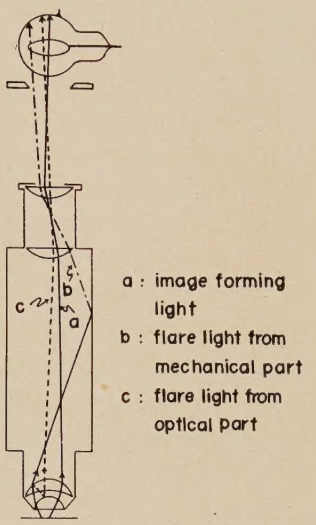

Fig. 1. Scheme of development of flare light.

It is not difficult to estimate quantitatively the effect of the flare light caused by multiple internal reflections of light at air-to-glass interfaces of the optical system, the flare caused by scattering being overlooked. Let a light flux of one unit (13) enter the optical system composed of m air-to-glass interfaces, all having the same average reflectance $\bar{r}$, then the part $(1-\bar{r})/\{1+(m-1)\bar{r}\}$ of the flux is transmitted, of which the part $(1-\bar{r})^m$ is utilized for imagery, the effect of scattering and absorption of light being neglected. Thus the ratio θ of the flare flux to the total transmitted flux is given by

$$\theta = \left\{\frac{1-\bar{r}}{1+(m-1)\bar{r}} - (1-\bar{r})^m\right\} \Big/ \frac{1-\bar{r}}{1+(m-1)\bar{r}}.$$

The values of θ for various values of m are calculated and shown in Table I, for $\bar{r}=0.05$ (for all usual optical systems) and $\bar{r}=0.01$ (for coated optical systems).

Now if the dimension of the luminous object is small compared with the distance from the object to the lens system, then the flare flux is widely spread over the image plane with a sharp image as its center, and hence the ratio of the intensity of illumination (i.e. light flux per unit area on the image plane) of the flare light to that of the sharp image is very much less than the above ratio θ. But as the dimension of the luminous object is increased with its brightness kept constant, the ratio in question gradually

TABLE I

Calculated values of amount of flare light for various numbers of air-to-glass interfaces.

m	θ (Per cent) for $\bar{r}=0.05$	θ (Per cent) for $\bar{r}=0.01$
1	0.00	0.00
2	0.25	0.01
3	0.72	0.03
4	1.40	0.06
5	2.26	0.10
6	3.27	0.14
7	4.44	0.20
8	5.73	0.27
9	7.12	0.35
10	8.62	0.43
11	10.20	0.52
12	11.84	0.62
13	13.54	0.72
14	15.29	0.84
15	17.09	0.97

increases, until for a sufficiently large luminous object it attains a saturated value which is just the same as θ. This saturation occurs when the diameter of the luminous object is of the same order of magnitude as the distance of the object from the lens system. Thus the ratio θ in Table I can be interpreted as the ratio of the intensity of illumination of the saturated flare light on the image plane to the actual intensity of illumination on the same plane, and the value of the ratio is 13.5 per cent in our microspectrophotometric apparatus with thirteen uncoated air-to-glass interfaces.

Now suppose a minute portion at the center of this large luminous object is blackend opaque. The behavior of the flare light originated from the outer parts of the object remaining unchanged, the central dark image is illuminated up by the flare flux, and hence if the transmittance of this portion is measured in these circumstances, the result would be θ in Table I, instead of zero. Thus the value of 13.5 per cent would be obtained for an absolutely opaque minute object in our apparatus.

Next suppose a minute portion at the center of the large luminous object have a light transmittance T. The flare light contributed from the outer parts of the portion is the same as before, i.e. θ when the outer intensity is

TABLE II

Correlation between true and measured transmittance.

True transmittance (Per cent)	Measured transmittance (Per cent)
0	13.5
0.1	13.6
1.0	14.4
10	22.2
50	56.8
80	82.7
100	100

The number of air-to-glass interfaces is 13.

taken as unity, while the light converging to the central image loses the same percentage θ of it by internal reflections, so that the central intensity amounts to $\theta + T(1-\theta)$. Thus the value of 14.4 per cent would be obtained in our apparatus for a deeply stained nucleus of true transmittance 1.0 per cent as seen in Table II.

This is the circumstance in which the S-V effect causes a serious error in the measurement of the transmittance of a minute object of low transmittance embedded in transparent blank, as is often encountered in microspectrophotometry, although the effect is slight in case of highly transmittant objects. Several examples of light transmittance of cells and tissues are shown in Table III.

The effect is further enhanced by the presence of the flare light due to scattering of light in the optical system, for which mathematical treatment is very difficult. But it is certain that the effect is more serious in lenses of smaller size, as in microscope objectives of high magnifying power, and that the effect increases rapidly as the number of air-to-glass interfaces increases. Indeed, the error in our example (13) mentioned above is higher than the value 14.4 per cent expected in Table III, and the difference can be attributed to the effect of the flare due to scattering.

Although the error due to the S-V effect is so serious as has been explained above, it is quite a simple matter to get rid of it. The effect of the flare light from both origins diminishes as the illuminated area including the minute portion is reduced, until it is almost completely eliminated when the illuminated area is included within the portion in question. Thus in all modern

TABLE III

Examples of light transmittance of cells and tissues for λ 5461 Å.

Cells of tissues	Animal	Staining method of Feulgen reaction	Transmittance § (Per cent)
Liver	Rat	M.F.*	46.7
Cerebellum	,,	,,	30.1
Adrenal	,,	,,	14.1
Pancreas	,,	,,	11.4
Liver	,,	,,	7.0
,,	,,	,,	5.9
,,	,,	,,	4.1
,,	,,	,,	2.4
First spermatocyte	,,	U.F.†	1.1
,, ,,	,,	M.F.	0.72
Embryonal tissue	Frog	U.F.	0.21

* M.F. means the Feulgen reaction modified by Sibatani and Naora (22, 24).
† U.F. means the usual Feulgen reaction.
§ This value shows an example of the transmittance of a single nucleus in the given tissue fixed in 50 per cent formalin.

microphotometers for measuring the density of photographic plates, the device is essential to condense the illuminating light flux within the very part to be measured. The same principle has been applied to our microspectrophotometric apparatus with successful results, as described below.

In this connection it should be remarked here on the relationship between the amount of the flare light and the aperture of the illuminating optical system. Theoretically, the amount of flare due to *optical parts* is quite independent of the aperture, and the facts are exemplified by the following simple experiments. The light transmittance of a single nucleus of a rat liver cell was measured by our apparatus, with various values of the cone angle of the illuminating light flux, the illuminated area being kept constant, i.e. 1 micron in diameter. The measured values of the transmittance coincide, as is shown in Table IV, within the accuracy of the measurement (14), which is chiefly determined by the reproducibility of illuminating the exact center of the spherical nucleus.

Another nucleus was measured with extended area of illuminated portion, i.e. 13.3 micron in diameter, with the result that our conclusion is the same, except that the measured values show a distinct enhancement from the true value, as the natural consequence of the S-V effect.

TABLE IV

Relation between cone angle of illuminating light flux and measured transmittances.

Diameter of illuminated portion: 1.0 μ		
N.A. of condenser lens	Cone angle	Transmittance (Per cent)
0.80	63°	44.4
0.97	79°	45.0
1.05	87°	44.8
1.19	102°	44.3
1.25	110°	44.6

Diameter of illuminated portion: 13.3 μ		
Cone angle	Transmittance (Per cent)	True transmittance* (Per cent)
63°	32.0	
79°	31.7	
87°	32.0	26.1
102°	31.6	
110°	32.4	

* True transmittance was measured under the condition that the diameter of the illuminated portion is 1.0 micron and the cone angle of the illuminating light flux is 110°.

If the *mechanical parts* of the measuring apparatus are carelessly designed, mechanical flare is added to the optical flare, and the combined both is sometimes called "glare" (16, 25). The main part of the mechanical flare is caused by the reflection of light at inner surfaces of tubes, and the amount increases with either the area of the illuminated portion or the aperture of the illuminating optical system, and hence the amount of "glare" is dependent on those factors. The mechanical flare can be removed, however, by careful designing of the mechanical parts, and does not deserve further discussions.

DESCRIPTION OF APPARATUS

Fig. 2 shows the outlook of our microspectrophotometric apparatus, of which a light source and a monochromator are out of view, while Fig. 3 shows schematically the arrangement of the optical system, the details of which are to be explained below.

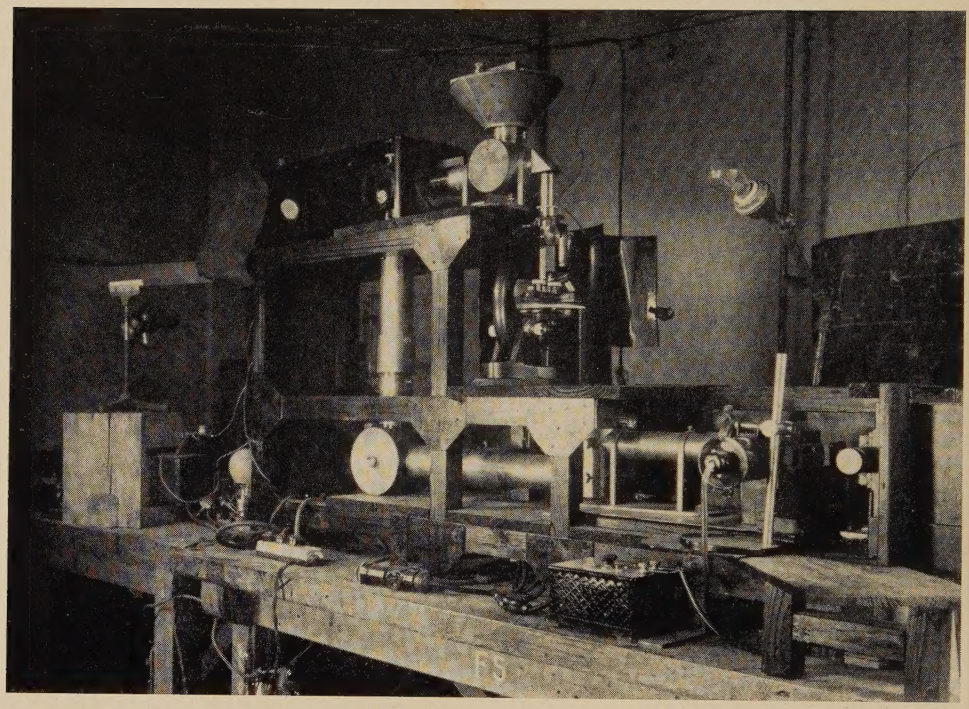

Fig. 2. Microspectrophotometric apparatus for measurement of light transmittance in visible region.

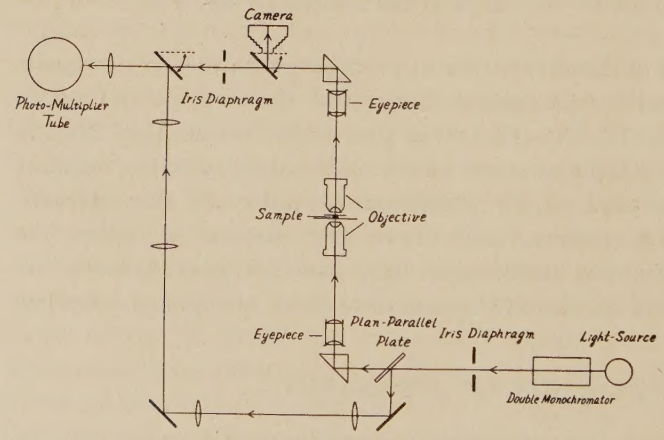

Fig. 3. Schematic diagram of the microspectrophotometric apparatus.

Illuminating system. The most remarkable feature of our apparatus lies in the optical system which illuminates distinctly the minute portion of the specimen the spectral transmittance of which is to be measured, and not the

Fig. 4. Special microscope for microspectrophotometry.

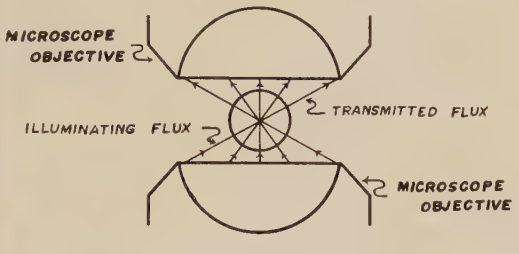

Fig. 5. Scheme of optical path through sphere.

wide area surrounding that portion, in order to eliminate the S-V effect. This can be done by focussing a uniformly illuminated aperture of an iris diaphragm, which serves as the secondary light source of variable diameter (2–10 mm), to the plane of the specimen, with a reduction of 1/2000, by a usual photomicrographic system with the reversed direction of light. Thus only the minute portion of the specimen, 1–5 micron in diameter, is uniformly and intensely illuminated.

A high power oil immersion objective, with a numerical aperture of 1.25–1.30, is used as the illuminating objective, as ordinary microscope condensers are not suitable because of their large residual aberrations, nor objectives of lower N.A. value because of their inferior definition for smallest images. And specimens are mounted on thin cover glasses instead of thick slide glasses, to match the short working distance of the high power objective.

It was shown by experiences that the illuminating optical system should have a fine focussing device as in ordinary microscopes, and also two-dimensional lateral movements in order to make the optical axis of the illuminating optical system coincide precisely with that of the magnifying system, and afterwards a special microscope with an illuminating system meeting these demands has been constructed, as shown in Fig. 4.

Magnifying system. The magnifying optical system, which throws a magnified image of the specimen to the image plane, is the same as an ordinary photomicrographic system, the magnification being 2000. Another iris diaphragm is so placed in the image plane as to coincide, with respect to both position and size, with the magnified image of the minute area in the specimen, which is again the reduced image of the first iris diaphragm, so that only the light flux passing through the second diaphragm can reach the photo-receiver, all extraneous stray light being cut off. Here again a high power, oil immersion objective (N. A. 1.30–1.40) is used, by the same reason as in the case of the illuminating objective.

It should be remarked here that our optical system is especially advantageous, other than the merit of eliminating the S-V effect, for the microspectrophotometry of nuclei with *spherical form.* When the reduced image of the first diaphragm is formed at the center of a small transparent sphere, and when the size of this image is sufficiently small compared with the diameter of the sphere, then, the length of the optical path of any beam of light passing through the sphere is the same, and is equal to the diameter of the sphere, being independent of the inclination angle of the beam against the optical axis, as shown in Fig. 5. Thus the circumstances are quite the same as in the case of the usual spectrophotometric analysis of solution kept in a cuvette with parallel plane windows. Therefore, if the sphere is made up of a homogeneous solution, we can determine exactly the concentration c of the solution by the formula

$$T = e^{-\varepsilon c (2r)},$$

or

$$c = \frac{\log_{10}(1/T)}{2\, r\, \varepsilon \log_{10} e}$$

by measuring the radius r of the sphere with an occular micrometer and the transimittance T of the sphere with our apparatus, provided the extinction coefficient ε of the solution be known. This theory has been experimentally ascertained (12, 14) for artificially prepared minute spheres of aqueous solution of safranin dispersed in cedar oil, with a limitation that the size of the reduced image of the light source should be smaller than one third the diameter of the sphere.

We can also determine the relative amount of the solute in each sphere of the same solution without the knowledge of the value of ε. The total amount of the solute in a sphere, which is the product of the concentration c into the volume $(4/3)\pi r^3$ of the sphere, is given as

$$\frac{2\pi}{3\varepsilon \log_{10} e} r^2 \log_{10}(1/T),$$

and the value $r^2 \log_{10} (1/T)$, or $r^2 E$, where E is defined as $E = \log_{10} (1/T)$, represents the relative amount of the solute in each sphere (14).

The circumstances may not be so simple in the case of spherical nuclei, as their contents are not perfectly homogeneous and hence light scattering may disturb the above theory. But the effect of this non-homogeneity is the less, the larger the cone angle of the illuminating light flux. Here we find another merit of our high power objective, the value 1.30 of N.A. corresponding to the cone angle as large as 108°.

Side tube. As shown in Figs. 2 and 3, a second optical path is set up beside the main path passing through the specimen in question, and either path is interchanged by 45° turn of a reflection mirror. The significance of this "side tube" is as follows.

When we measure the transmittance of a specimen, we measure at first the light flux through the specimen, and then that through the "blank" by laterally sliding the slide glass; and the ratio of the two fluxes gives the required transmittance of the specimen in case when the intensity of the light source does not change throughout the measurement.

If it is not sure that the intensity of light is kept constant, the measurement should be repeated, since it is rather troublesome to bring back the same specimen again to the exact center of the microscopic field.

We measure repeatedly the ratio of the light flux through the specimen to that through the side tube, with easily reproducible way of turning the mirror, and then similarly the ratio of the light flux through the blank to that through the side tube, and get the required transmittance of the specimen as the ratio of the two ratios. No error is introduced by the change of the intensity of the light source during these two series of experiments. This method is especially useful in the measurement of a number of nuclei on the same slide glass, as we can measure them successively without an utmost care to the constancy of the light source throughout the measurement, and also without referring to the blank in each time.

The side tube system has another merit. We put a neutral tint light absorber in the side tube, the transmittance of which is so chosen that the light flux through the side tube is approximately the geometrical mean of the light flux through the specimen and that through the blank. By this process the accuracy of the measurement is improved especially for deeply stained nuclei of low transmittance of light, say of 0.1 per cent (14).

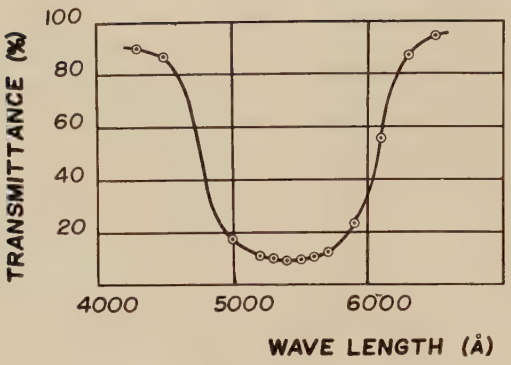

Fig. 6. Spectral-transmittance of Feulgen-DNA complex produced by Feulgen reaction F.

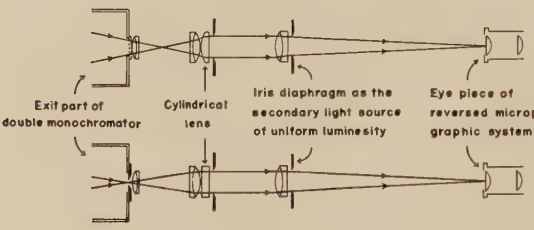
Exit part of double monochromator
Cylindrical lens
Iris diaphragm as the secondary light source of uniform luminesity
Eye piece of reversed microphotographic system

Fig. 7. Scheme of optical system between exit part of double monochromator and reversed microphotographic system.

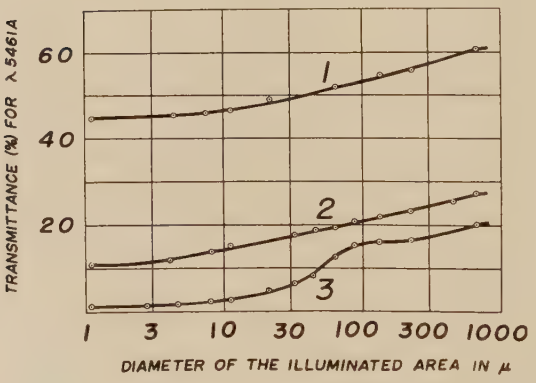

Fig. 8. Relation between various sizes of illuminated area and transmittances.
1. Isolated nucleus of rat liver (diameter, 6.2 micron);
2. Isolated nucleus of rat liver (diameter, 4.4 micron);
3. First spermatocyte of rat testis (diameter, 5.5 micron).

Photo-receiver. A photo-multiplier tube of RCA 931A is used as the photo-receiver, whose photo-current is measured by a galvanometer with the current-sensitivity of 3×10^{-10} Å. The linear relation between the deflection of the galvanometer and the light flux is checked by measuring the transmittance of the same specimen with various levels of the intensity of the light source, with the result that the error due to the failure of the linearity is less than one per cent of the measured value throughout our measuring range.

Light source. In microspectrophotometric analysis of DNA content in

animal nuclei, we stained them by Feulgen reaction modified by Sibatani and Naora (15, 22, 24) and the transmittance of the Feulgen-DNA complex formed by the reaction is measured with monochromatic light of a definite wave length.

In order to find the most suitable wave length for the purpose, microspectrophotometric survey is made for nuclei thus stained, with the result that the minimum of the transmittance, or the maximum of the absorption, lies always in the wave length region between 5400Å and 5500Å in the case of F and MB staining (24), as shown in Fig. 6.

Very fortunately the strong green light of wave length 5461Å from mercury arc lies just in that region, and it is decided to use this light throughout our work. Thus an ultra high pressure mercury arc lamp, 100 V-750 W, with brightness of 30,000 cd/cm^2 is used as the light source, and the light of 5461Å is selected by a double monochromator.

A simple optical system, as shown in Fig. 7, is used to illuminate uniformly the circular aperture of the iris diaphragm in Fig. 3, by the light from the exit slit of the monochromator.

COMPARISON OF RESULTS OBTAINED BY APPARATUS WITH AND WITHOUT S-V EFFECT

As described above, our microspectrophotometric apparatus is characterized by its being free from the S–V effect. But it is also quite easy to cause the S-V effect of any degree of magnitude appear in our apparatus, by adding an auxiliary optical system to our illuminating system and thus illuminating the circular area of an arbitrary diameter with the specimen at its center. A simple experiment has been made with the purpose to check our theory about the S-V effect discussed on pp. 260–265.

Three nuclei of spherical form, 4–6 micron in diameter and differing in the transmittance of light were chosen as the specimens, and their transmittance was measured by our apparatus. Each spherical specimen was illuminated by discs of light having center in common with the sphere and diameters varying from 1 to 650 micron, while a fixed diaphragm of 2 mm in diameter was placed at the image plane of the magnifying optical system of 2000 × magnification, thus leading to the photo-receiver the light corresponding to the small portion of the specimen 1 micron in diameter. The results are shown in Fig. 8. In cases of small illuminated areas, the measured values of the transmittance of the specimens are constants which must be considered as the true transmittance of the nuclei. When the illuminated

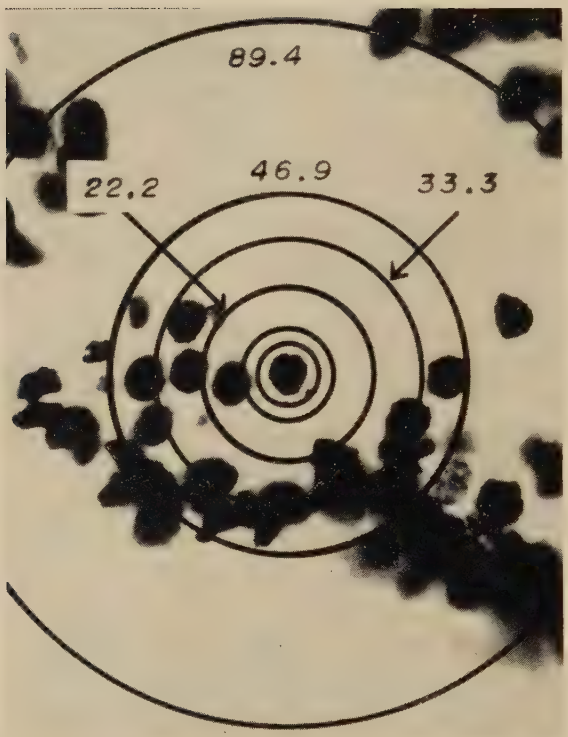

Fig. 9. Specimen No. 3, a first spermatocyte of rat testis, lies at the center. Number attached to each circle indicates its diameter in micron.

area is broadened, the measured values show a gradual increase, but they tend to constant saturated values, which must be the sum of the true value and the flare light discussed above. Although the maximum diameter 650 micron in our experiment is not sufficient to cause the fully saturated flare, the enhanced value exceeds the flare ratio shown in Table I, where we can attribute the difference to the flare caused by the scattering of light. The irregularity of the curve for specimen No. 3 can be interpreted as the effect of other nuclei existing in the same field, as shown in Fig. 9.

These results indicate that the true value of the transmittance can be obtained only when the diameter of the illuminated area is less than (preferably one third) the diameter of the nucleus.

Now it may happen to be argued that the enhancement of the light transmittance of nuclei due to the S-V effect, or the decrease of the DNA content in the nuclei, never lead to erroneous biological conclusions so long as the *relative values* of the DNA content among nuclei are concerned. But this argument is not correct, because the decrease of the DNA content does not

TABLE V

Comparison of true transmittance and true amount of DNA, with erroneous values due to *S-V* effect.

T (%)	0.1	1.0	5.0	10	20	40	70	100
$\frac{T_{S\text{-}V}}{T}$	136	14.4	3.56	2.22	1.54	1.20	1.06	1.00
$\frac{M_{S\text{-}V}}{M}$	0.289	0.421	0.576	0.654	0.732	0.799	0.841	1.00

appear in the same proportion for each nucleus, as the error in the transmittance due to the S-V effect is of the additive character.

These circumstances can be made clear by the following considerations about the simplest case. Let the light transmittance of perfectly spherical nuclei be measured by our apparatus in both conditions without, and with fully saturated, S-V effect, and their relative DNA content be calculated by the formula r^2E in both cases. Further, the flare due to internal reflections only is taken into consideration, that due to scattering being neglected. The values of enhanced transmittance are already shown in Table II, and the ratios of $T_{S\text{-}V}$ to T, i.e. the enhanced value to the true value are calculated for several values of T, and shown in the upper half of the Table V. Now the corresponding ratio of the DNA content, decreased $M_{S\text{-}V}$ to true M, is given as

$$\frac{M_{S\text{-}V}}{M} = \frac{r^2 E_{S\text{-}V}}{r^2 E} = \frac{E_{S\text{-}V}}{E} = \frac{\log_{10}(1/T_{S\text{-}V})}{\log_{10}(1/T)},$$

and the calculated ratios are shown in the lower half of the same table. From these figures it is clear that the proportion of the decrease of the DNA content due to the S-V effect is not the same, but is the more marked, the lower the light transmittance of the nucleus is.

It may be pointed out in this connection, that the formula r^2E may not be adequate to evaluate the relative DNA content in cases accompanied by the S-V effect. Indeed, various formulas, including the r^2E formula (19), have been proposed by many investigators, say Swift (25) and Lison (9), etc. By adopting any other formula, however, our conclusion that the proportion of the decrease of the DNA content due to the S-V effect is not the same for every nucleus, is unaltered.

TABLE VI

Relation between relative DNA content of liver cells and first spermatocytes measured with and without *S-V* effect.

Cell	With *S-V* effect		Without *S-V* effect	
	No. of measured nuclei	Mean of relative DNA content (r^2E) and standard deviation	No. of measured nuclei	Mean of relative DNA content (r^2E) and standard deviation
Liver cell	28	5.48 ± 0.85	25	6.05 ± 0.69
First spermatocyte . .	10	4.98 ± 0.76	10	10.14 ± 0.67

Hence for example, two spherical nuclei of equal DNA content and of different size, hence of different light transmittance, must be observed as being of different DNA content in the presence of the S-V effect, and possibly *vice versa*. The importance of this fact can hardly be exaggerated, especially in discussions concerning to the constancy *versus* variability of the DNA content in individual nuclei from the same species of cell, and with the purpose to examplify the above conclusion, the following three series of experiments have been performed.

Throughout these experiments nuclei of spherical form were chosen as specimens, which were illuminated just in the same manner as in the previous experiment.

Experiment 1. Nuclei of liver cells and of the first spermatocytes of a young rat, fixed in 50 per cent formalin and stained with the usual Feulgen reaction, were employed as specimens, and their light transmittance was measured by our apparatus with the illuminated areas of 9 and 1 micron in diameter, of which the former does, and the latter does not, involve the S-V effect, although the effect is still not very large in the former case. As seen in the reduced results shown in Table VI, the presence of the S-V effect always causes a significant lowering of the values of the DNA content, and the proportion of the decrease is not same and is more marked in the value of the spermatocyte nuclei which have a very low transmittance (1–2 per cent) due to their remarkably intense staining. These facts conform to our theoretical conclusions stated above.

Experiment 2. An assemblage of rat liver nuclei, fixed and stained in the same way as in the Experiment 1, were used as specimens, and their transmittance was measured with and without the S-V effect just in the same

manner as in the previous experiment. The reduced results are shown in the left half of Table VII and as numbered histograms in Fig. 10.

Without the S-V effect, out of 22 nuclei measured 17 form the first group with the lowest DNA content, 4 the second with the medium and 1 the third with the highest value. On introducing the S-V effect, the order of the individual nuclei with respect to their DNA content is altered irregularly, and three

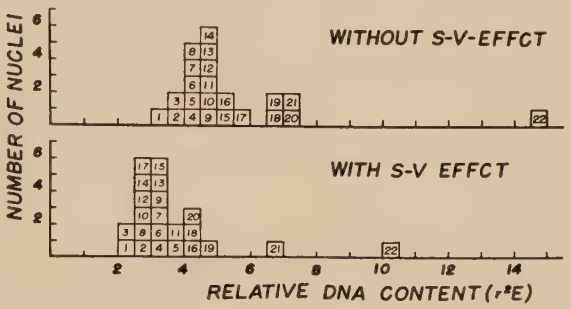

Fig. 10. Comparison of histograms of relative DNA content of liver nuclei of a rat determined through microspectrophotometry with and without S-V effect. The corresponding numbers in two histograms indicate measurements on the same nucleus.

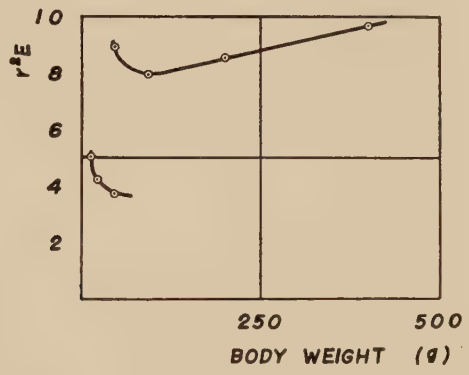

Fig. 11. Relation between DNA content (r^2E) of liver cell nuclei and growth of rat.

nuclei Nos. 18–20 are shifted from the second group to the first, in which the variance of the DNA content is greater than in the true first group, while the nucleus No. 21 retains its position in the second group. Such result can be said to be the natural consequence of our theory, as the transmittances of the nuclei Nos. 18–20 amount to 10–15 per cent and that of No. 21 as high as 22.3 per cent, and the decrease of the DNA content due to the S-V effect is the more marked, the lower the transmittance of the specimen is.

This deformation of distribution can never be remedied by the adoption of the other formula for calculating the DNA content, say Swift's formula (25) instead of r^2E. The circumstances are essentially unchanged, as shown in Table VII.

TABLE VII

Comparison of relative DNA content calculated by Swift's and Naora's formula from the transmittances measured through the apparatus with the *S–V* effect.

Group	Calculated by			
	r^2E		Swift's formula	
	No. of measured nuclei	Mean of relative DNA content and standard error	No. of measured nuclei	Mean of relative DNA content and standard error
First	20	3.34 ± 0.14	20	2.16 ± 0.10
Second	1	6.75	1	4.39
Third	1	10.38	1	5.81

Experiment 3. Liver cell nuclei, taken from a mature and an immature rat, the body weight of which is 395 gram and 11 gram respectively, were fixed with 50 per cent formalin and stained by the usual Feulgen reaction, and their light transmittance was measured by our apparatus with the illuminated area of 1, 15 and 560 micron in diameter. The error due to the S-V effect is null in the first case, medium in the second, maximum in the third case.

The relative amount of DNA content in each nucleus measured was calculated by the formula r^2E, and the result is shown in Table VIII. The true values of the mean relative DNA content are given, by the measurement with the minimum illuminated area, as 8.50 and 4.51 for the mature and the immature rat respectively, the value of the ratio being as high as 1.88. As the illuminated area is broadened, or as the error due to the S-V effect increases, the value of the relative DNA content as well as the ratio of them decreases, until for the maximum illuminated area of 560 micron in diameter, the ratio is nearly unity, which may seem to correspond to the theory of the constancy of the DNA content during the growth of a rat, a mice or other animals, as reported by Pollister (17, 18), Swift (25) and others (4).

By eliminating the S-V effect in their microspectrophotometric measurement, however, the author and Sibatani (15) have found a rhythmic variation of DNA content in liver cells of rats during their growth, as shown in Fig. 11, which shall be fully explained in another report.

The above three experiments may suffice to prove our important propo-

TABLE VIII

Correlation between relative DNA content of liver cells during growth.

Diameter of illuminated area (μ)	Liver cell of rat (body wt. 11 g)		Liver cell of rat (body wt. 395 g)		$\frac{r^2 E_{(395\,g)}}{r^2 E_{(11\,g)}}$
	No. of measured nuclei	$r^2E \pm$ S.E.	No. of measured nuclei	$r^2E \pm$ S.E.	
1	28	4.51 ± 0.12	27	8.50 ± 0.17	1.88
15	29	3.91 ± 0.09	29	7.27 ± 0.17	1.86
560	20	2.80 ± 0.08	44	3.17 ± 0.17	1.13

sition, that the S-V effect brings about not only the error in the value of transmittance of a nucleus, but also the error in the relative values of a content among nuclei, and hence leads to biological conclusions which are erroneous, to say nothing of inaccurate.

Finally a remark should be given that throughout the above discussions and experiments our microspectrophotometric apparatus with thirteen uncoated air-to-glass interfaces was dealt with. With antireflection coated optical parts, the amount of flare due to internal reflection is greatly reduced, as shown in Table I, and hence also the error due to the S-V effect. But the flare due to scattering can never be reduced, and at best the optical flare amounts to ca. 2 per cent, as recently reported by Lison (11), which is still verly large for the measurement of deeply stained specimens of transmittance value less than 1 per cent. Further, the anti-reflection coating is not effective for lights of all wave length range, and our conclusion concerning the S-V effect is never altered for an apparatus with the most carefully coated optical parts.

Thus we may conclude that the correct result in microspectrophotometric works can only be obtained by the use of equipments which are free from the S-V effect.

SUMMARY

The microspectrophotometric equipments hitherto employed for cytochemical analysis are not free from the Schwarzschild-Villiger effect (S-V effect), and the measured results contain errors which are sometimes so serious as to mislead to biological conclusions deduced from the measurements. The magnitude of the error due to the S-V effect and its influence on

the determination of the DNA content in cell nuclei were theoretically estimated, and the theory was ascertained by several experiments.

An improved optical system for microspectrophotometry was designed and constructed with succesful result to eliminate the S-V effect completely, and its details were described.

The author wishes to extend his sincere thanks to Prof. Dr. Z. Koana for advising him regarding the Schwarzschild-Villiger effect and the method for preventing it, and for guiding him throughout his investigation. He is also indebted to Dr. A. Sibatani, of Department of Cytochemistry and Histology, Yamaguti Medical School, Ube, for the preparation of the biological specimens, to Dr. G. Kuwabara for valuable advice about photometric techniques, and to Miss K. Yosiwara for her assistance.

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Experimental Cell Research, **8**, *279–304 (1955)*

ACUTE COLD-INDUCED CHANGES IN CELLULAR PROTEIN AND NUCLEIC ACIDS IN RAT LIVER AND BRAIN

A DESCRIPTION AND CRITIQUE OF BIOCHEMICAL AND CYTOCHEMICAL STUDIES[1]

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Received May 11, 1954

During the past several years the authors have been investigating effects of brief cold exposure on the liver and supraoptic nuclei of the hypothalamus. Conventional cytochemical and biochemical techniques have been used to measure the complex phenomena encountered. Because gross discrepancies between the several types of data have appeared, it has been necessary to analyze some of the facts, inferences, and assumptions upon which these particular experimental procedures are based. This examination has brought into focus a number of physiologic and chemical factors which are frequently ignored in the analysis and interpretation of such information, for example, the extreme lability of the intact organism to brief stress, and the rapid fluctuations in total organ mass under like circumstances. The observations made in clarifying some of these discrepancies have revealed several physiologic mechanisms operating during brief cold exposure which otherwise might have escaped notice. The study has clearly accented the fact that each of the several techniques involved can give unique information of importance concerning one or several phases of such a biological problem but that a more definitive understanding of the processes involved inevitably demands a multifacetted approach. It is hoped that this report may prove of value in the future interpretation of similar data and may, in addition, point the way toward a more accurate understanding of certain cellular responses of the animal under stress.

This communication is not intended to be a critique of the technical limitations of the relevant biochemical or cytochemical methods. Excellent studies such as those of Glick, Engström and Malmström (37) provide much

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protein values. Selection of valid standards is of importance if these data are to have any quantitative significance. The use of identical reference values for the cytoplasmic nucleic acids and proteins of both hepatic parenchymal cells and supraoptic neurons may be an unwarranted simplification. Amino acid analysis and characterization of brain proteins reported by Block (5, 6), and of liver proteins by Fürth and coworkers (35), Luck (50, 51), Michel and Moutte (54), and Gjessing (36), offer some support for this simplification as regards the protein standards. Marshak (53) has reported large variations in the molar ratio of uracil/adenine between nuclear and cytoplasmic RNAs of a number of organs though he found the ratio of thymine/adenine to be relatively constant for corresponding nuclear DNAs. Chargaff and associates (20) had previously reported similar variations for cellular RNAs from a variety of species and from different organs within the same species. Whether the total ultra-violet absorptive capacity of such RNAs fluctuates with base ratios has not been indicated by these studies. This capacity is presumably a function of the total absorbing chromophores and their resonance states. The influence of isolation procedures on the latter factor will be discussed shortly.

The data of Table I indicate that the concentration of cytoplasmic protein of both liver cells and supraoptic neurons increases under the following conditions: a) brief cold exposure in the intact fed animal; b) 18–24 hour fast in the intact animal; and c) bilateral adrenalectomy in the fed animal. The observed data suggest a decrease in concentration of cytoplasmic nucleic acid in both cell types following cold exposure of fed, intact animals. Fasting

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TABLE I

Mean integrated ultra-violet extinction values for cytoplasm of frozen-dried liver cells and supraoptic neurons of control albino rats and litter mate cold-exposed animals under various conditions. Per cent cytoplasmic protein and nucleic acid calculated from these microabsorption data.

No. Number of animals/total number of integrated mean cytoplasmic values (approximately 75–150 separate values integrated in each).
E_{255} = Mean $E_{255} - E_{325}$ (5 μ frozen-dried section). E_{275} = Mean $E_{275} - E_{325}$ (5 μ section). Subscript numbers in parenthesis = standard deviation of mean.
% Prot. and % N. A. in grams $\times 10^{-14}$/cubic micron equals grams/100 cc tissue.
Superscript 1) 2) 3) 4) under Liver – % Prot., Control Fed, refers to values for four different sets of standard extinction values viz. 1) N.A. $E_{255} = 0.125$, $E_{275} = 0.079$; Protein: $E_{255} = 0.0033$, $E_{275} = 0.0051$. 2) Same values for N.A., protein: $E_{255} = 0.0035$, $E_{275} = 0.007$. 3) N.A. $E_{255} = 0.088$, $E_{275} = 0.054$; Prot. $E_{255} = 0.0033$, $E_{275} = 0.0051$. 4) Same N.A. values as 3) and Protein: $E_{255} = 0.0035$, $E_{275} = 0.007$. All remaining % values calculated from following standard equations:

$$E_{255} = \text{M N.A.} \cdot 0.107 + \text{M. Prot.} \cdot 0.0034,$$
$$E_{275} = \text{M N.A.} \cdot 0.067 + \text{M. Prot.} \cdot 0.0061,$$

where M N.A. and M. Prot. are grams $\times 10^{-14}/\mu^3$ of tissue for nucleic acid and protein respectively.

TABLE I

Exper. condition	Control										One hour cold exposure									
	Liver					Supraoptic neurons					Liver					Supraoptic neurons				
	No.	E_{255}	E_{275}	% Prot.	% N.A.	No.	E_{255}	E_{275}	% Prot.	% N.A.	No.	E_{255}	E_{275}	% Prot.	% N.A.	No.	E_{255}	E_{275}	% Prot.	% N.A.
Fed	5/13	0.30 (.08)	0.26 (.08)	(1) 22	1.8	5/19	0.26 (.08)	0.20 (.07)	9	2.1	4/10	0.26 (.09)	0.29 (.09)	31	1.5	3/6	0.25 (.08)	0.25 (.08)	23	1.6
				(2) 14	2.0															
				(3) 23	2.5															
				(4) 15	2.8															
				17	2.2															
18–24 hr. fast	6/16	0.34 (.11)	0.36 (.14)	38	1.9	2/4	0.20 (—)	0.24 (—)	28	0.5	5/22	0.26 (.16)	0.29 (.16)	31	1.5	1/6	0.51 (.03)	0.57 (.03)	62	1.9
Bilat. adrenalect. Fed + 1% NaCl	3/12	0.37 (.11)	0.38 (.13)	38	2.2	3/16	0.31 (.18)	0.29 (.17)	23	2.2	3/18	0.17 (.11)	0.15 (.09)	11	1.2	3/13	0.33 (.13)	0.26 (.10)	14	2.5

does not appear to alter the concentration of nucleic acid in liver, though it seems to in supraoptic neurons. In the fasted animal the cytoplasmic protein concentration of liver does not change significantly with brief cold exposure. One isolated study of supraoptic neurons in the fasted rat suggests an increase in cytoplasmic protein concentration with cold. Cold exposure of bilaterally adrenalectomized rats is accompanied by a decrease in concentration of liver cytoplasmic nucleic acid, and the reverse is noted for supraoptic neurons of the same animals. Conventional t-tests for estimating the significance of differences between means have been employed in evaluating the microabsorption data of Table I. The relevance of such methods for complex data of this type is debatable. It is interesting to note, nonetheless, that for supraoptic neurons no mean values differ at better than the 5 per cent probability level, while for liver cytoplasm differences between mean values are significant at the 5 per cent level or better for control and cold exposure data, as well as for controls studied under the three stated conditions.

BIOCHEMICAL AND RELATED STUDIES

Protein estimates. In a series of 17 experiments on the effect of brief cold exposure, control rat livers had a mean blood-free protein concentration of 18.0 g per cent (sigma 0.65) while blood-free livers from cold-exposed animals contained 21.4 g per cent protein (sigma 0.4). These and other values will be found in Table III. The trend indicated by the cytochemical data is confirmed in this instance, though the absolute concentration change cytochemically is far in excess of that noted chemically. A few chemical analyses of whole brain and of cerebral cortex under the same conditions failed to confirm changes observed cytochemically in supraoptic neurons.

Centrifugal fractionation of sucrose homogenates of liver provided further support for the conclusion that cytoplasmic liver proteins increase in concentration with brief cold exposure. A typical run is summarized in Table II,

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Table II

A typical differential centrifugal fractionation of sucrose homogenates of liver and cerebral cortex in fed control and 1 hour cold exposed litter-mate Wistar rats. W.H.: Whole homogenate (0.25 M. sucrose). N.F.: Nuclear fraction (600 g for 10″ in 0.25 M. sucrose, 2 additional washes in 0.88 M. sucrose with resedimentations at 600 g for 10″). M.F.: Mitochondrial fraction (8500 g for 10″ in 0.25 M. sucrose, 2 additional washes in 0.88 M. sucrose with resedimentations at 11,000 g for 30″). Mic. F.: Microsomal fraction (19,000–23,000 g for 1 hour in 0.125 M. sucrose in isotonic saline, 2 runs). S.F.: Supernatant fraction. Numbers in parenthesis indicate individuals means of duplicate aliquots. All values expressed as grams or milligrams per 100 grams of original wet weight of tissue.

TABLE II

Tissue homog. and experimental condition	W.H.			N.F.			Mito. F.			Mic. F.			S.F.			% Recovery		
	N, g %	DNA-P mg %	RNA-P mg %	N, g %	DNA-P mg %	RNA-P mg %	N, g %	N, % tot.	RNA-P mg %	N, g %	N, % tot.	RNA-P mg %	N, g %	N, % tot.	RNA-P mg %	Tot. N	Tot. RNA-P	Tot. DNA-P
Liver control fed (2 animals)	2.64 (2.62–2.66)	23.3 (22.8–23.7)	102.7 (102.7–102.7)	.413 (.407–.420)	22.3 (21.8–22.8)	5.6 (5.5–5.7)	.578 (.571–.585)	21.9	14.8 (13.9–15.7)	.537 (.537–.537)	20.4	54.2 (51.9–56.5	1.08 (1.06–1.10)	40.9	20.9 (20.6–21.2)	98.8	93.3	95.8
Liver-fed 1 hour cold (2 animals)	3.07 (3.05–3.10)	22.3 (21.8 22.8)	111.0 (110–112)	.415 (.388–.441)	22.4 (22.2–22.5)	4.42 (4.37–4.46)	.595 (.572–.598)	19.3	15.9 (15.1–16.6)	.700 (.630–.769)	22.8	57.8 (57.4–58.1)	1.35 (1.31–1.38)	44.0	29.2 (26.9–31.4)	99.8	97.5	100
Brain cortex control fed (2 animals pooled)	1.73	2.56	31.2	.097	2.40	3.63	.356	21.0	6.85	.658	38.0	12.9	.574	33.0	13.4	97.5	118.0	93.8
Brain cortex 1 hour cold (2 animals pooled)	1.59	2.85	32.8	.100	2.52	3.31	.347	20.8	6.45	.618	39.0	15.0	.541	34.0	13.3	99.0	116.0	88.5

and includes comparable data for rat cortex of the same animals. From this Table it can be seen that there is an increase in the percentage of total homogenate protein to be found in the microsomal and supernatant fraction of cold-exposed liver. Mitochondrial and nuclear fractions show a slight relative decrease. All of these values are expressed as concentrations and the inherent limitations of this expression will be discussed. Of particular interest is the fact that the microsomal fractions of liver and brain cortex of cold-exposed, as compared with control rats were no longer as completely sedimentable at 23,000 × g, either before or after the addition of dilute sodium chloride to the sucrose medium. Except for this, brain cortex homogenates of cold-exposed animals did not differ significantly from those of control rats. None of the trends observed for supraoptic neurons cytochemically could be verified with these biochemical techniques in cortex homogenates. Some of the problems involved in the interpretation of such data are considered in critical studies previously mentioned (27, 29).

Nucleic acids. Chemical analyses failed to reveal any decrease in concentration of RNA in cold-exposed liver (see, e.g., Table II). On the contrary, brief cold exposure caused a significant increase in concentration of RNA (approximately 15 per cent), represented largely in the microsomal and supernatant fractions, where similar protein changes are observed. Only in the nuclear fraction of sucrose homogenates were nucleic acid changes noted which were consistent with cytochemical analyses (not reported here). In fractionated cerebral cortex a slightly higher proportion of total RNA-P was recoverable from the microsomal fraction of cold exposed animals, as in liver, but no significant differences were noted in the supernatants.

Calculations of intracellular substances from biochemical and other data. The ratio of cellular to extracellular phases was determined in frozen dried, stained, liver sections from blocks used for the cytochemical studies already described. Control absolute values for hepatocellular and extracellular phases were obtained by multiplying total wet liver weights (mg wet liver/g body weight) by these directly determined ratios. Data obtained from a study of four control male rats are plotted in Fig. 1. Comparable curves were plotted for litter-mate cold-exposed animals. Similar ratios have been determined for

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TABLE III

Fluctuations in total organ mass, and in total content and concentration of certain reference substances, under a variety of experimental conditions. Control and experimental animals of authors' series. Harrison (40), Weil and Groat (70), carefully matched as regards body weight, sex, and environmental conditions.

TABLE III

Control						Experimental					
Organ and condition	Total organ mass, g	Concentration g or mg/100 g wet tissue		Total organ content		Organ and condition	Total organ mass, g	Concentration g or mg/100 g wet tissue		Total organ content	
		Protein g %	DNA mg %	Protein g	DNA mg			Protein g %	DNA mg %	Protein g	DNA mg
Rat liver (fed—Authors' series)	9.0	18.0	235	1.62	21.2	Rat liver (fed, one hour cold—Authors' series)	7.65	21.4	236	1.64	18.1
Rat liver (fed—Harrison 40)	8.91	18.0	210	1.61	18.7	Rat liver (24 hr. fast—Harrison 40)	7.03	22.2	270	1.56	19.0
Rat liver (fed—Novikoff *et al.* 56)	—	19.45 (calc.)	200	—	—	Rat liver (fasted—Novikoff *et al.* 56)	—	24.4 (calc.)	310	—	—
Rabbit liver (fasted?—Lowe *et al.* 48)	56 (mean value from data)	21.4 (calc.)	73.4 (calc.)	11.9 (calc.)	41.0 (calc.)	Rabbit liver (cortisone—25 mg I.M./day for 3 days. Lowe *et al.* 48)	98 (mean value from data)	11.8 (calc.)	30.1 (calc.)	11.6 (calc.)	29.5 (calc.)
Rat brain (fasted?—Weil and Groat 70)						Rat brain (fasted? bilat. adrenalect.—Weil&Groat70)					
			Total P.		*Total P.*				*Total P.*		*Total P.*
Male	1.791	—	322 mg	—	5.72 mg	Male	2.027	—	312 mg	—	6.32 mg
			Total P.		*Total P.*				*Total P.*		*Total P.*
Female	1.779	—	342 mg (calc.)	—	6.09 mg	Female	2.009	—	315 mg (calc.)	—	6.33 mg (calc.)

bilaterally adrenalectomized rats before and after cold exposure, but liver wet weight measurements are thus far too few to permit calculation of these absolute compartment values. Nuclear diameters of control and cold-exposed liver cells were measured directly on ultra-violet photomicrographs registered at 257 mμ, magnification 1250 ×.

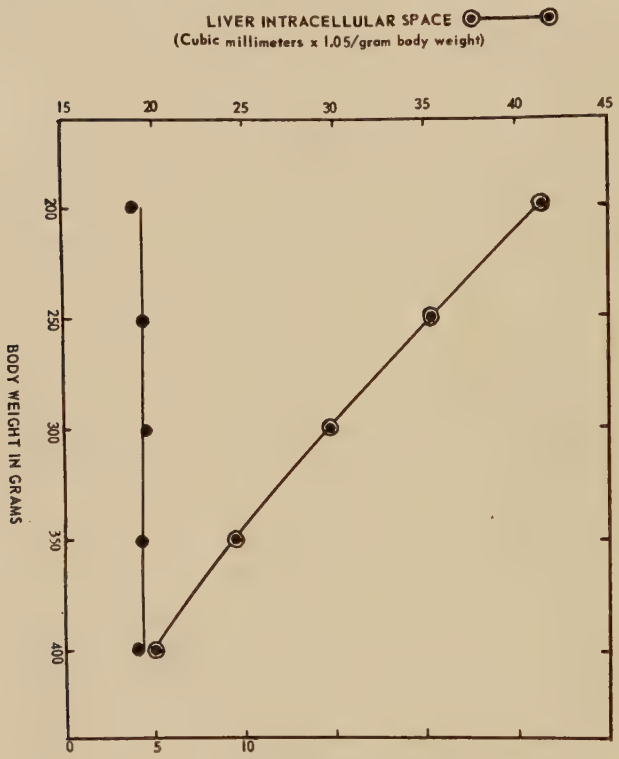

Fig. 1. Total intracellular and extracellular space of liver expressed in mm^3 per gram body weight, for male control rats of 200 to 400 gm. Values for intracellular space referred to left ordinates, those for extracellular space referred to right ordinates. Values must be divided by 1.05 (assumed density of liver tissue) to give mm^3 directly.

The values of Table III A are calculated utilizing cytological data mentioned above as well as chemical data from analysis of whole liver homogenates and their particulate and non-particulate fractions (Figs. 1, 3 and Tables II, III). It should be emphasized that the wet weight of rat liver in one hour cold-exposed animals averages 15 per cent less than control values. The mean calculated volume of liver cell nuclei is 12 per cent less in cold-exposed than in control animals. Control liver cell volume for calculating nuclear/cytoplasmic ratios is taken from Harrison's data (40). It is evident from Table III A that the cytoplasmic concentrations of protein and RNA increase measureably and proportionately with brief cold exposure, whereas there

TABLE III A

Calculated mass, volume, and chemical components of the various compartments of liver in the control and acutely cold-exposed rat. See text, Figures 1, 3, Tables II and III for reference data.

Compartment or value	Control (Mean values: 300 g male rat) Mg/ g body weight	Mm³/ g body weight*	Mg/ 100 mm³ **	One hour c-old exposure (Mean values: 300 g male rat) Mg/ g body weight	Mm³/ g body weight*	Mg/ 100 mm³ **
1) Total liver mass . . .	34.5	32.8		29.3	27.9	
2) Total hepatocellular phase	29.8	28.4		26.2	25.0	
a) Cytoplasmic compartment	27.1	25.8		23.9	22.8	
b) Nuclear compartment	2.7	2.6		2.3	2.2	
3) Extracellular phase .	4.7	4.5		3.1	2.9	
4) Cytoplasmic protein .			20.8			24.4
5) Cytoplasmic RNA . .			1.3			1.5
6) Nuclear protein . . .			24.6			30.3
7) Nuclear NA			3.85			4.0
a) RNA			0.75			0.7
b) DNA			3.10			3.3

* $\text{Mm}^3/\text{g body weight} = \frac{\text{Mg/g body weight}}{\text{Tissue spec. dens.}}$, where

Tissue specific density is assumed to be 1.05 throughout (41).

** Mg/100 mm³ is mg component per 100 mm³ of specific compartment. This value is, of course, identical with Gm per 100 cc or with the cytochemical concentration data of Table I, expressed as Gm $\times 10^{-14}$ per cubic micron of tissue.

is no significant deviation from control values in the total amount of either protein or RNA (concentration × total compartment volume per g body weight).

The factors chosen for the above computations are in good agreement with recent published values of Harrison (40, 41) and others. Hogeboom *et al.* (43), Muntwyler (55), and Novikoff (56) have published liver fractionation data with which the chemical values of Table II (control) are consistent. Assuming, nonetheless, that the chosen factors are still only first approximations, the fact remains that the ultra-violet microabsorption

measurements of cold-exposed liver cells (Table I) indicate a much greater increase in cytoplasmic protein concentration than can be accounted for biochemically. Even more obtrusive is the paradox that cytochemical measurements indicate a decrease in concentration of cytoplasmic nucleic acid of liver (and supraoptic neurons) with cold exposure, while the reverse is observed with biochemical methods.

'Native' ribosenucleic acid. In an attempt to explore the paradox mentioned above with regard to cytoplasmic nucleic acid, 'native' RNA was isolated from rat liver by the technique of Grinnan and Mosher (38). The yield was approximately 200 mg/100 g wet liver. The original preparations reported by them were studied in the ultracentrifuge from which data they concluded that the probable molecular weight of their materials was in excess of 300,000. Two similar protein-free fractions isolated in this laboratory contained 6.3 and 6.7 per cent phosphorus (their fractions varied from 6 plus to 7 plus per cent P). The ultra-violet absorption spectrum of the former fraction is shown in Fig. 2, curve 1. On the same graph, curve 2, is the absorption spectrum of a commercial protein-free RNA (Nutritional Biochemicals Co. 8.4 per cent P) in equal concentration. It is evident that the commercial preparation absorbs substantially more than the 'native' rat liver fraction. The E 1 mg per cent per cm per mg P at 255 mμ is itself 10 per cent higher for the commercial preparation than for the 'native' RNA. Gentle alkaline hydrolysis at 37° C over a period of 21 hours produces a gradual increase in ultraviolet absorption of this 'native' RNA, as indicated in curves 1 a, 1 b, 1 c, and 1 d of this figure. Standard nucleic acid extinction values customarily used (17, 57, 58) are consistent with values for a commercial preparation such as shown in Fig. 2, curve 2. The literature is replete with analyses of similar preparations (59, 60). For this reason the ultraviolet absorption characteristics of Grinnan and Mosher's preparation are of particular interest. Tsuboi (68, 69) has isolated sodium salts of DNA (9.0 per cent P) and RNA (9.4 per cent P) by more vigorous procedures from mouse liver. Strenuous alkaline or enzymatic hydrolysis of his preparations was accompanied by an increase of as much as 30 per cent in in the ultraviolet absorption within the selective range. Tsuboi's preparations absorbed equivalently to commercial RNA prior to such hydrolysis. The evidence suggests, therefore, that a 1 cm layer of RNA may, at pH 7.0, have an $E^{1\text{ mg per cent}}_{255\text{ m}\mu}$ ranging from 0.088 to approximately 0.16 depending on its degree of polymerization and perhaps on many other factors as well. Currently no data are available to indicate what specific standard value should be used and under what circumstances.

Since a hydrolysis and differential precipitation of DNA from RNA is involved in both the Schmidt-Thannhauser and Schneider techniques any differences in native cellular RNA due to state of polymerization will be partially eliminated. Moreover, since there is such a wide range of phosphorus values for various RNA preparations, the concentration or amount of any

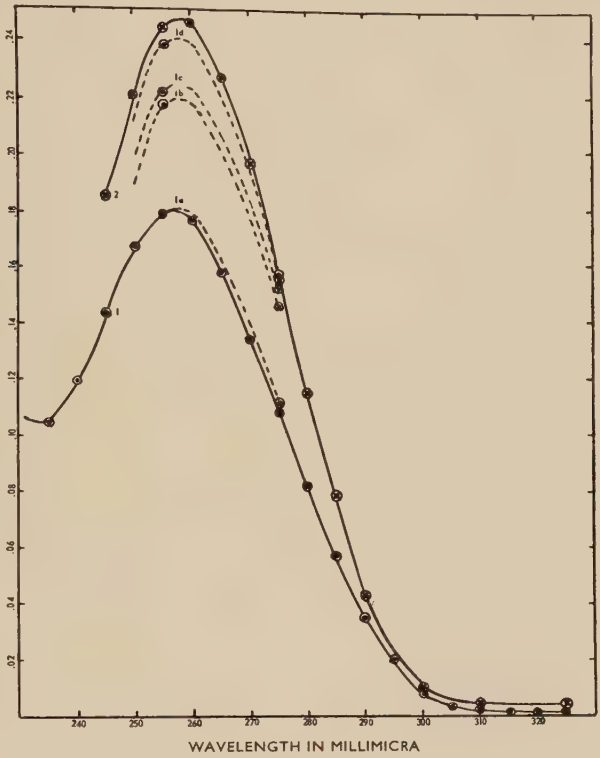

Fig. 2. *Ultraviolet absorption spectra of 1 mg per cent solutions of native rat liver RNA (curve 1) and commercial RNA (curve 2, National Biochemicals Co.) in phosphate buffer at pH 7.0.* Curves 1a through 1d are absorption spectra of 1 mg per cent solutions of native rat liver RNA following alkaline hydrolysis in 1 N. NaOH at 38° C for 1 minute (1a), 25 minutes (1b), 115 minutes (1c) and 21 hours, 45 minutes (1d). There was no detectable change in E at 325 mμ during hydrolysis for total of 46 hours. Hydrolyzed preparations diluted with borate-citrate buffer and adjusted to final pH 7.0. Readings in Model DU, Beckman Spectrophotometer.

particular tissue RNA can only be inferred from phosphorus data. A more satisfactory solution to the chemical dilemma is to be found in some such procedure as estimating specific purine and pyrimidine components of DNA and RNA by a chromatographic technique such as Marshak (53) has used with encouraging success.

Concentration versus amount. In many biochemical and cytochemical studies, the amount of substances is expressed in mass units per standard volume or arbitrary mass referent as, e.g., g or mg per 100 g wet weight or some convenient multiple thereof. The significance of physiologic alterations in concentrations expressed in such terms, bears closer scrutiny.

TABLE IV

Effect of varying periods of cold exposure on the concentration and total amount of rat liver protein, with per cent deviation of observed values from experimental, control and inter-litter normal trend (Fig. 3). Duplicate determinations.

Experimental condition	Rat body weight (g)	Liver protein concentration (g/100 g wet weight of liver) per cent	Mg total liver protein per g body weight	Per cent deviation of total protein from observed controls	Per cent deviation of total protein from control trend (Fig. 3)
Control	236	18.8	6.5	0	−10.3
15″ cold . . .	259	18.1	7.6	+20.5	+10.2
30″ cold . . .	216	19.4	7.6	+11.6	+ 1.31
45″ cold . . .	246	20.0	7.05	+10.3	0
60″ cold . . .	233	20.1	6.55	+ 0.3	−10.0
75″ cold . . .	232	19.2	6.78	+ 3.8	− 6.5

* Protein calculated as total organ N. × 6.25. Maximal contributions of non-protein nitrogen to these values is as follows: Control: NPN–194 mg per 100 grams wet liver. Nucleic acid nitrogen — 173 mg per 100 grams wet liver. 60″ cold: NPN–220 mg per 100 grams wet liver. Nucleic acid nitrogen — 187 mg per cent. Total non-protein nitrogen contributes up to 20 per cent to total liver nitrogen change with cold.

An analysis of the data obtained under conditions of brief cold exposure, which is qualitatively prototypical of a wide variety of stressful circumstances (e.g. painful manipulation, anesthesia, fright, or fatigue), is particularly revealing. It has been noted that after one hour of brief cold (Tables I, II, III A, IV) there is a measurable increase in concentration of proteins in liver cells and supraoptic neurons. The fact is, however, that for liver the overall shrinkage of that organ and its total cellular compartment explains the chemically measured concentration change. Thus liver, as an organ, contains no more protein nor nucleic acid after 1 hour of cold exposure than before. Cytochemical data suggest that the same concentration shift is true for supraoptic neurons of cold-exposed animals, though pertinent chemical data are not obtainable with present techniques. One might be tempted to interpret the observed increase in protein concentration with cold as a manifestation of protein synthesis if the net protein change were not known. It is possible, of course, that a true charge of protein to liver does occur during the very early phases of cold exposure. Table IV indicates that such may actually be observed within the first 45 minutes of cold exposure. It is equally clear from this table that the noted changes in protein concentration do not reflect total increases or decreases in any consistent way.

A variety of different physiologic and pathologic factors may produce

substantial alterations in the total wet weight and chemical composition (concentration-wise) of liver and other organs. Additional data culled from the literature for rat and rabbit liver as well as for rat brain are summarized in Table III. Fluctuations in brain composition are particularly interesting since, in the adult animal, this organ is considered to be relatively fixed in mass and composition in contrast, e.g., with liver. Table III shows that fasting or brief cold exposure may produce a substantial increase in concentration of liver protein and DNA without significant change in the total amounts of these components. The data of Harrison (40, 41) and Novikoff (56), summarized in this Table, indicate an increase of about 25 per cent in concentration of rat liver protein with a 24 hour fast. Thus, again, the trend indicated by the ultra-violet microabsorption data of Table I for the fasted rat is confirmed although the magnitude of the concentration change appears to be far greater from the cytochemical measurements than can be explained by chemical analysis.

Change in total liver mass and composition are even more impressive in the rabbit treated with relatively large doses of cortisone for three days. In this instance, also, total organ protein is not decreased, though concentration shifts might seem to indicate this. The fact is that if Lowe's mean values (48) are analyzed in terms of the trends diagrammed in Fig. 3 (assuming that growing rabbits show changes similar to those in growing rats) there is probably an increase in total liver protein of cortisone treated animals. (Lowe's control animals were, on the average, very much heavier than his treated animals. The difference far exceeded that referable to weight losses usually noted under similar conditions with cortisone therapy). Calculation of these and the other protein data of Table III for unit mass of DNA tends to clarify such concentration shifts in terms of probable cell composition. Such a correction is used effectively by Harrison (40, 41). The completeness of separation of DNA from RNA even under ideal control conditions, let alone under rapidly changing physiologic states, is open to question (29). This will be discussed later. In the bilaterally adrenalectomized rat (Table III) brain wet weight increases postoperatively. Under these circumstances total phosphorus of brain also increases but the concentration of phosphorus in mg per 100 grams fresh brain decreases. Undoubtedly these alterations in concentration in brain and in liver as well reflect in greatest measure shifts in water and in available compartment volumes. Concentration data alone serve to obscure many significant aspects of the true picture. Our preliminary observations in bilaterally adrenalectomized male rats indicate a minimal decrease in wet weight of whole brain postoperatively. With brief cold ex-

posure of such animals wet weight of brain increases slightly over both control intact and non-cold exposed operated brain values. Thus since brief stress would appear to alter these organ values significantly, the precise physiologic state of the animal at time of sacrifice and during the immediately preceding 'control' period should be carefully regulated if meaningful data

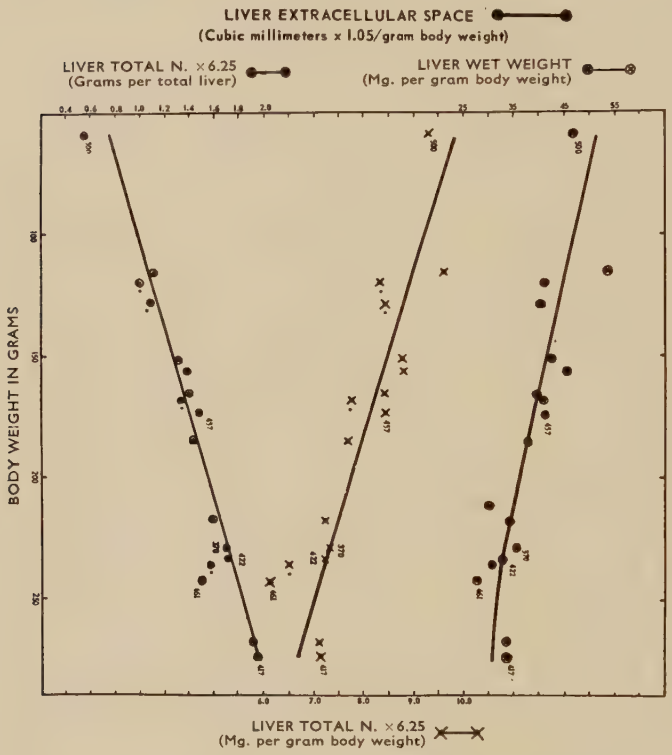
LIVER EXTRACELLULAR SPACE
(Cubic millimeters x 1.05/gram body weight)
LIVER TOTAL N. ×6.25
(Grams per total liver)
LIVER WET WEIGHT
(Mg. per gram body weight)
BODY WEIGHT IN GRAMS
LIVER TOTAL N. ×6.25
(Mg. per gram body weight)

Fig. 3. Whole organ data for liver in fed, female control albino rats of Wistar strain, each rat of separate litter origin. Superscript numbers refer to adrenal ascorbic acid in mg/100 g adrenal in those rats where this determination was performed. * indicate values from aliquots of lyophilized liver stored in a vacuum dessicator. All other determinations on fresh liver homogenates in KCl.

are to be obtained. Such rigid controls are also indicated in the handling of intact animals since seemingly insignificant stress may cause changes in wet liver mass and total protein content (see below).

Suggested biological reference standards. Age, body weight, and sex are further factors of significance to be considered in studies designed to assay rapid changes in organ weight and composition. It is often difficult to obtain control and experimental groups whose mean body weights agree closely. Where precise matching is not possible 'control' curves such as represented in Figs. 3 (liver) and 4 (brain) are of distinct value. These indicate that total organ nitrogen bears a roughly linear relation to body weight within the

range 60–300 g for female rats. Similar trends are obtained for male animals though the absolute values are approximately 15 per cent higher for males. Wet organ weight and total organ nitrogen in brain both expressed in mg per gram body weight, follow a paraboloid course during this active phase of growth. In liver and brain, deviations from the mean trend in

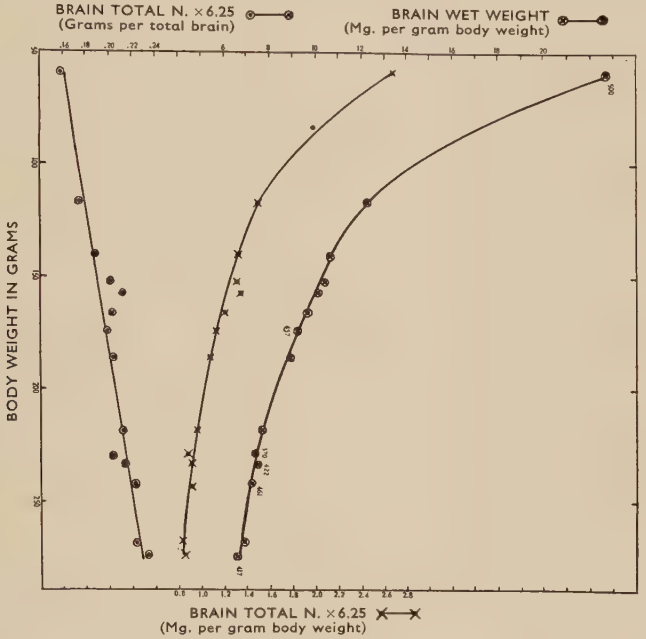
BRAIN TOTAL N. ×6.25
(Grams per total brain)
BRAIN WET WEIGHT
(Mg. per gram body weight)
BODY WEIGHT IN GRAMS
BRAIN TOTAL N. ×6.25
(Mg. per gram body weight)

Fig. 4. Whole organ data for brain (transected at inferior margin of pons) in fed control female albino rats of Wistar strain for which liver data are summarized in Fig. 3. Notations as in that fig.

control animals sacrificed under similar conditions, are approximately equivalent, percentagewise. These deviations are of such magnitude that comparable values obtained under known experimental conditions are perhaps most conservatively evaluated in terms of per cent deviations from this trend rather than as variations from the intrinsic controls of the immediate study. From such reference curves corrections can be made directly for small variations in body weight inevitably encountered even among litter-mates of the same sex. Table IV summarizes data evaluated on the basis of experimental, control, and inter-litter control trend values. Such control curves serve an eminently useful purpose in providing a background grid of "spontaneous" variation against which any presumed experimental change should be read. Adrenal ascorbic acid variations in these animals suggest the possibility that these control fluctuations may, in part, be manifestations of unrecognized 'minor' stress. The trends observed (Table IV)

with brief cold exposure in repeat experiments when intrinsic control values are used, point to a real change under this circumstance, but the projection of this change against background variation among inter-litter controls indicates that it is of only slightly greater magnitude than that accompanying unrecognized and uncontrollable environmental changes or variations to be anticipated from intrinsic differences between individuals.

DISCUSSION

An analysis of the cytochemical and biochemical information. The ultra-violet microabsorption technique used permits precisely accurate intra-cellular localization of measurements made. Thus the values for cytoplasm summarized in Table I are unequivocally cytoplasmic values whether the organ be liver or an autonomic neural center. Were it possible, in every instance, to determine the appropriate ultra-violet absorption reference standards for the substances studied, and under conditions identical with those in the cell section measured, there could be no question of either the quantitative or qualitative validity of such cellular measurements. Reference standards determined under such conditions are, however, not available nor is it likely that they could all be defined in the predictable future. Studies are now in progress in this laboratory to explore a number of factors which appear to be critical in this regard. It is hoped that this information may make it possible to derive more valid reference standards.

The biochemical techniques used suffer from limitations of different types. Adequate computational standards do not exist, to be sure, for such a substance as cellular RNA, but this problem is avoided by recording concentrations or amounts in terms of phosphorus or nucleotide components. Nonetheless such analyses involve a selective separation of chemical components from tissues or fractions. Profound changes in these biological substances may, and undoubtedly do, occur during the process of separation and isolation. The cytochemical data reported here may reveal certain qualitative changes in RNA as it exists in the cell, but if this be true then data have little or no quantitative significance. On the other hand, the biochemical methods, in principle, give accurate quantitative information but may completely obscure qualitative changes of considerable biological importance. Thus, it has been demonstrated that RNA, isolated and purified by different procedures, varies not only in its ultra-violet absorptive capacity but also in phosphorus content (and very probably in degree of polymerization).

The fact that brief cold exposure produces a significant increase in the

concentration of liver RNA-P is in direct contrast to the observation that the ultra-violet microabsorptive characteristics of liver cell cytoplasm, under like circumstance, are consistent with a considerable decrease in concentration of RNA. Several possibilities exist to explain this discrepancy.

1) The particulates contributing in large measure to cytoplasmic absorption in liver may change in size, density, chemical composition, or scattering properties following brief exposure to cold. It was observed that the microsomal fractions of liver (and brain cortex as well) no longer sedimented well at 23,000 × g, as similar fractions did in controls. This change might reflect an alteration in the state of polymerization of either the microsomal RNA, protein or both. That substantial changes in ultra-violet absorptive capacity would accompany such a change is suggested by the data of Fig. 2, and by other observations mentioned in that section. The following thesis appears plausible: in the functioning liver cell (or neuron) an equilibrium exists between polymerized RNA-protein and depolymerized RNA-protein, the former low in phosphorus and absorbing less in the ultra-violet, the latter high in phosphorus and absorbing more intensely in the ultra-violet. With such acute stress as brief cold this equilibrium is shifted to the left. The result is that the specific ultra-violet absorptive capacity decreases although the concentration of substance remains the same or even increases slightly. Under the conditions of chemical analysis, possible alterations in composition inherent in such a shift might be obscured. As a product of such a shift, phosphate would be liberated intracellularly. The fact that ribonuclease can potentiate such polymeric changes and that this enzyme is known to exist in most cells makes feasible such a shifting reaction. If this situation were the true one the apparent contradiction in biochemical and cytochemical data would be explained.

2) On the other hand, the apparent decrease in cytoplasmic nucleic acid concentration of liver with cold, cytochemically, may reflect merely a decrease in the scattering potential of such cytoplasmic particles. Sedimentation data seem to be inconsistent with such an assumption since cold-exposed microsomes are less dense (or smaller) than control microsomes. It may well be that the estimate of scattering registered at 325 mμ with the quartz monochromatic objective, N.A. 1.25, corrected for 275 mμ is not sufficiently precise to reveal small but significant changes in light scattering. No measureable scattering could be detected in the frozen-dried tissues from control or cold-exposed intact animals. The definition in the measured photographic image at lambda 325 is quite poor with the large effective condenser aperture (adjusted to give full and even illumination of the im-

mersion field) used in this study. Sharply defined images were regularly recorded at 257 and 275 mμ with the quartz monochromatic immersion objective and identical condenser aperture. Significant scattering was noted in the liver cells of bilaterally adrenalectomized animals, hence gross changes can be detected. The only available data, therefore, indicate that non-selective absorption is not measurably decreased with cold exposure, hence that this explanation is not the probable one.

3) Possible changes in orientation of absorbing RNA may occur with cold exposure. The isolation and verification of Grinnan and Mosher's highly polymerized RNA establishes such a possibility for RNA, as it has long been recognized for DNA. A number of theoretical (23) and experimental (66, 71) studies have been concerned with this factor. Thorell and Ruch (66), for example, found that errors due to changes in orientation of DNA are quite small for transmittance values of 50 per cent or more. In the transmittance ranges encountered in 4 micron sections of frozen-dried tissues (Table I) small errors might be expected on the basis of their work. Since a crystalline quartz prism was interposed in the microabsorption system employed in this study, the illuminating beam was undoubtedly partially polarized, but the same is true of the beam of the Beckman spectrophotometer used for the macrochemical studies of Thorell and Ruch. No convincing data affirm the operation of such a factor in tissue RNA estimates by this microabsorption technique, but this possibility remains.

To return to the chemical data and their interpretation, it is usually impossible to specify the precise cellular origin of any particular substance analyzed, whether protein, nucleic acid or other. Brain is composed of neurons and their processes, a variety of glial elements, endothelial cells, histocytes, vascular and supporting membranes, a protein-rich extracellular ground substance, and finally, a complex extracellular compartment. All of these components make their contribution to brain homogenates. Fractions such as can be obtained by differential centrifugation will be characteristically heterogeneous. Neither in whole brain nor in any dissected fractions of brain are single cell types encountered. It is therefore not surprising that neither brain cortex homogenates nor their fractionated components confirmed trends noted cytochemically in supraoptic neurons. Homogenates of the hypothalamic area could not be successfully fractionated though homogenate nitrogen concentration was decreased in cold-exposed as compared with control animals. A similar change was observed in cerebral cortex (Table II). Because of the obvious heterogeneity of these preparations no direct comparisons with cytochemical information are possible. The

similarity of sedimenting characteristics of the microsomal fractions of brain and liver in cold-exposed animals is, however, of considerable interest in the light of the previous discussion. Differential centrifugal fractionation of whole rat brain homogenates (8, 9) provide data quite unlike those obtained in our analyses of rat cortex and serve to emphasize the impressive heterogeneity of this organ. Thus, extreme caution must temper interpretation of variations observed in brain or cortex fractions under the same or different physiologic conditions.

Chemical data on whole liver and its separable fractions cannot be compared directly with cytochemical data, without reservations. Liver, too, is not a homogeneous organ. Routine histologic study confirms this. Stowell (64), for example, reports that only about three fifths of the cells encountered in normal mouse liver are hepatic parenchymal cells. The remainder are endothelial and mesenchymal elements, ductal epithelial elements, and lymphocytes. Liver is, in addition, compartmentalized and its total extracellular compartment is fluctuant in volume and composition. Because of the known cellular heterogeneity of liver, studies on isolated liver nuclei or on nuclear fractions should be viewed reservedly, particularly when compared to cytochemical studies. Many such analyses have been published (53, 28, 3, 10, 41). It is probable that serious difficulties in the interpretation of such data will become increasingly apparent when critical comparisons are attempted between nuclei obtained under varying physiologic conditions. Reliance upon liver DNA values and fluctuations as an index of varying hepatocellular population, suggested, for example, by Davidson and Leslie (25), and used by Harrison and coworkers (40, 41) and Mandel *et al.* (52) among many others, is to be questioned when applied under significantly different physiologic conditions. Possible problems in quantitatively separating DNA from RNA under such circumstances have already been alluded to.

Compartment measurements (Fig. 1) do indicate that, in the rat, the bulk of hepatic mass is hepatocellular, even though non-hepatocellular nuclei may make up two-fifths of total nuclei. This was equally true for intact fed, fasted, and bilaterally adrenalectomized rats. The ratio of cellular/extracellular compartment remained remarkably constant under varying conditions. Thus chemical estimates of liver RNA and protein are good first approximations of hepatocellular RNA and protein. Minor corrections may improve the closeness of this approximation (Table III A) though they make no substantial difference. Cytochemical data obtained during this study indicate an increase in concentration of liver cytoplasmic protein in the in-

tact animal with brief cold, and brief fast and, in addition, point to a marked increase over control values following bilateral adrenalectomy. Of particular interest is the fact that these changes are accurately mirrored in supraoptic neurons where biochemical validation of any sort is not possible by current procedures. Chemical data obtained confirm the fact but not the magnitude of protein increase with cold exposure in liver. Other chemical investigations (Table III) show that the concentration of liver protein is increased with brief fast and also that large doses of cortisone (30, 48) produce reverse effects from those observed by us in bilaterally adrenalectomized animals. Preliminary chemical analyses of whole liver and brain in chronic, bilaterally adrenalectomized female rats indicate a 12–20 per cent increase in concentration of total liver nitrogen over controls, with a scarcely measurable change in the same direction for brain. There are, thus, general areas of agreement between these chemical and cytochemical protein data, a fact emphasized in more general terms several years ago by Catchpole and Gersh (20). Under all of the conditions studied cytochemical data consistently indicate changes greater than those chemically determined. This discrepancy may appear because the photographic ultra-violet microabsorption technique is not sensitive enough to permit even semi-quantitative estimates of cellular protein, as Caspersson has mentioned (14). A previous cytochemical study of motor neurons (59) demonstrated reasonably good agreement between ultra-violet and X-ray microabsorption data, predominantly for cellular protein. Nonetheless, the discrepancies noted are not of sufficient moment to demand more extensive exploration even though factors influencing the nucleic acid estimates may be operative here too, though in limited degree. In a very real sense this must be true, since the observed extinction values are the sum of both protein and nucleic acid contributions (as well as non-specific elements) at the wave length used.

The nature and significance of chemical changes. The data reported and reviewed should serve to point up some limitations inherent in chemical information couched in concentration terms. Needless to say, many standard cytochemical techniques use such a reference base. Cellular values as, for example, those of Hydén (45), Hydén and Hartelius (46) and those reported by one of us recently (57) are quite difficult to interpret critically for this reason. Though it is tempting to infer either protein synthesis or loss (or related chemical change) from concentration measurements, such inferences are rarely justified from concentration data alone (see Table III). This is true not only for animals of approximately equal development studied under varying conditions, but likewise for animals of different developmental

stage when such problems are particularly complex (34). Analysis of stained tissue sections (2, 18, 33, 72) is subject to the same errors in interpretation, no matter how sophisticated or sensitive the microcolorimetric procedure may be. Such a limitation is likewise pertinent for chemical data derived from tissues or fractions of tissues, as has been shown.

With highly labile substances like glycogen, concentration changes may be so great as to overbalance gross organ weight shifts. Resultant data may be of unquestionable significance. It is known, for example, that liver glycogen is almost completely depleted during a 24 hour fast (26, 40), and this change would be manifest whether expressed in total organ content or in concentration. In muscle, on the other hand, glycogen is not so labile under similar circumstances. Alterations in concentration, here, would have to be interpreted in the light of water shifts and alterations in available compartment volumes (see Hegsted *et al.* (42), and Lowry and Hastings (49)), and might best be referred to unit nitrogen, DNA or similar standard.

Under certain circumstances one can circumvent concentration distortions. The procedures described here for whole organ analyses accomplish this end. Cellular metabolic phenomena can be studied by a technique which permits single cell analysis in an organism whose metabolic events are confined within its own cell membrane. Numerous investigations from the Carlsberg Laboratory, of which that of Andresen and Holter (1) is but one example, exploit such a technique. Where one must study the complex interplay of integrative and modulating mechanisms characteristic of higher organisms such techniques can rarely be applied. Recently, Caspersson and his associates (15, 16) described an automatic integrative scanning apparatus which may be used conveniently in obtaining necessary cumulative data, thus intrinsically correcting for cellular compartment fluctuations. Edström (31), in Hydén's laboratory, has developed an admirable microanalytic method for individual large cells, specifically neurons. It is doubtful, however, that his method is applicable to neurons since the cell processes disrupted in his isolation procedure actually make up much of the bulk of the whole cell. Nonetbeless this represents a pioneer effort in the right direction.

Analysis of the incorporation of isotope-labeled substances into tissue components gives direct evidence of metabolic activity and is independent of fluctuations in organ mass. Borsook (7) has recently reviewed much meaningful information obtained by this technique. It is evident, however, that the significance of such 'turnover' studies for net synthesis is a function of the total amount of the labelled substance or fraction, not its concentration. Muscle 'proteins', for example, are reported to turn over quite slowly, yet

a small fraction of this protein turns over with impressive rapidity. P^{32} uptake studies of the nucleic acids of brain can be misleading for a different reason, as shown by Deluca and his coworkers (27). The problem here is one of isolating these substances from non-nucleotide P^{32}-labeled materials which turn over quite rapidly. The serious problem of specifying the exact cellular origin of many labeled fractions, particularly in neural tissue, limits conclusions which can be drawn from studies such as that of Samuels and his associates (27).

In spite of the many errors and limitations mentioned, much meaningful information concerning biochemical events at the cellular level can be obtained. It is doubtful, however, whether some of this information can be validly interpreted without specific awareness and understanding of referants used and of the effect of changing physiologic circumstances on these referants.

SUMMARY AND CONCLUSIONS

1. In the fed rat exposed to low temperature for one hour, there is an increase in concentration of protein and a decrease in concentration of RNA in the cytoplasm of liver cells and supraoptic neurons, as estimated by the photographic ultra-violet microabsorption technique.

2. Biochemical analyses of whole rat liver homogenates and of differentially sedimented fractions show an increase in concentration of protein, restricted to microsomal and supernatant fractions, with one hour of cold exposure. The concentration of RNA, under this circumstance, increases proportionate to protein and the change is manifest in the microsomal and supernatant fractions. Similar analyses of brain cortex and hypothalamic areas do not confirm concentration shifts indicated by the cytochemical data. Areas of disagreement are discussed.

3. Evidence from whole organ analyses indicates that the concentration shifts described are not associated with any significant change in total amount either of protein or nucleic acid. Shrinkage of total available cellular compartment explains concentration shifts. A number of similar phenomena are reported and discussed.

4. It is demonstrated that concentration shifts may, under a variety of commonly encountered circumstances, not accurately reflect changes in total amounts of substances present.

5. Chemical experiments are reported which indicate that there is a total, if transient, increase in whole liver protein during the first 45 minutes of

cold exposure, though this change is not reliably reflected in concentration measurements.

6. Some chemical and ultra-violet absorptive characteristics of a 'native' RNA isolated from rat liver are described. It is suggested that cytoplasmic RNA exists in an unstable equilibrium between highly polymerized and relatively depolymerized forms in the functioning cell. Reasons for postulating such an equilibrium are presented and certain factors as, for example, brief external stress, presumed to shift this equilibrium are mentioned. The possible biological significance of such an equilibrium in the role of a labile intracellular phosphate pool is proposed.

7. Cytochemical evidence indicates that the concentration of cytoplasmic protein in liver cells and supraoptic neurons is increased with brief fast and following bilateral adrenalectomy. Pertinent chemical information for liver is reviewed and evaluated. The effects of brief cold exposure on liver cells and supraoptic neurons of such animals, as inferred from cytochemical data, are presented.

8. Some serious limitations inherent in cytochemical and biochemical data, couched in concentration terms, are reviewed, and a number of available techniques for circumventing these limitations are discussed.[1]

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[1] The authors wish to express their indebtedness to Miss Barbara Hunt, Mr. Pope Hill, and Mr. Elliott Purlson for their valuable technical assistance. Mr. Philip Rhodes generously assisted in statistically evaluating cytochemical data.

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Experimental Cell Research, **8**, *305–328 (1955)*

THE ALTERATION OF INTRACELLULAR ENZYMES

I. YEAST CATALASE AND THE EULER EFFECT[1]

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Received May 20, 1954

When the enzyme catalase is extracted from the erythrocyte, it behaves in certain ways unlike the original intracellular enzyme (20). When crystalline beef liver catalase is unfolded at the air/water interface and then compressed into a solid insoluble fiber, it behaves unlike the soluble enzyme from which the fiber was prepared (21). Curiously, the surface-unfolded and the intracellular catalases behave qualitatively like one another whilst the soluble and extracted enzymes also behave qualitatively like one another. The first two differ from the latter two in that (i) their specific activity is much lower, (ii) they are activated by short pre-treatments of heat (50–56° C), and in that they are much more resistant to inactivation by (iii) prolonged heat and (iv) ultraviolet (UV) irradiation.

Since it is unlikely that interfaces of the air/water type of extremely high interfacial energy, can exist within living cells, I advanced the working hypothesis that intracellular catalase exists adsorbed and partially unfolded at an interface of the oil/water type (20); following extraction of the enzyme or lysis of the cell, the enzyme was visualized as having been desorbed from the intracellular interface, becoming 'rolled-up' into a more active configuration, resembling that of the crystalline catalase in solution. This *interfacial hypothesis* has proven to be of considerable heuristic value, although critical evidence in its favor has not yet been adduced.

A series of lesser known papers by Hans von Euler and collaborators, (10, 11, 12, 13) extending over a thirty-year period, encouraged me to attempt the test of my hypothesis in the study of yeast catalase. Euler had shown that

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[1] Certain aspects of this work have been described in papers presented to the Nova Scotian Institute of Science (22) and to the American Physiological Society (23).

[2] This work has been supported from its inception in 1950 by the National Research Council (Ottawa), and lately additional support has been forthcoming from the National Cancer Institute (Canada).

[3] This paper is dedicated to Professor Hans v. Euler, whose work, cited *in extenso* below, was the foundation upon which these experiments were based; his comments and encouragement have been deeply appreciated.

pretreatment of yeast cells with (*a*) heat, (*b*) drying, (*c*) 'protoplasmic poisons' (including chloroform and toluene), and (*d*) UV, resulted in a marked increase in the activity of the intracellular catalase; more recently, he has demonstrated the existence of a similar situation in plant cells. This phenomenon, that is, *the marked increase in activity of intracellular catalase following treatment of the cell with various physical and chemical agents*, I propose to designate the *Euler effect*.

Such increase in the activity of an intracellular enzyme might *a priori* be ascribed to one or more of the following factors:

1) an increase in permeability of the cell membrane to substrate molecules;

2) inactivation of a chemical inhibitor present initially within the cell;

3) release within the cell of some activating agent;

4) increase of the actual amount of enzyme present;

5) change in intrinsic properties of the enzyme itself, resulting from the direct or indirect action of the agents used on the structure of the enzyme; such a change in intrinsic enzymatic properties I propose to call *enzyme alteration*.

Euler himself has not attempted an experimental attack on the mechanism of the Euler effect, although in his early papers he leaned in favor of possibilities *1* and *2* above; there are, however, certain statements in his papers which are not inconsistent with the intrinsic change of possibility *5*.

The purpose of this paper is twofold; (i) to rule out possibilities *1–4* above, hence adducing evidence in favor of enzyme alteration as the cause of the Euler effect, and (ii) to demonstrate that the intracellular catalase, having undergone enzyme alteration, acquires a wholly different set of associated properties, causing it to become qualitatively identical in behaviour to the extracellular, extracted catalase in solution, which must also, therefore, have undergone enzyme alteration.

EXPERIMENTAL

General.—Enzyme activity was determined as the slope of the linear portion of the curves relating μl oxygen evolved to time (see Fig. 2), as previously described (21). The lag phase in these curves does not enter into the activity determination. The activities presented in the data below should be multiplied by the factor 40 to yield velocity constants in μl per minute. A circular Warburg apparatus was used, the bath being maintained at 30°, with excursion and rate of shaking as described elsewhere (21). Shaking was not interrupted during reading of the manometers.

Baker's (Fleischman's) yeast was used throughout, the methods of washing and

handling being those of Aldous (1). Cells were plated out on 'Difco' wort agar medium; in each experiment where plate counts are reported 2 series of dilutions were run each with 3 or 4 plates and the mean used for the experimental points. Colonies were counted by means of a simple, electrically operated contact counter.

After the final preparatory washing, the wet yeast was weighed out on a semi-micro balance and made up as a one per cent suspension in Sorensen's phosphate buffer at pH 7.2. Despite the undoubted variability in degree of hydration from day to day, the small variation in number of cells as determined from colony counts, direct counts in the Levy counting chamber, and enzymatic activity did not interfere with either results or conclusions.

In the reaction vessel, 1 ml of the yeast suspension to be used was placed in the Warburg vessel proper, and 1 ml of the substrate in the sidearm. Conditions will be specified for each experiment, but generally the 1 per cent yeast suspensions were diluted with buffer either 50 or 200 times before use in the respirometer. The thermobarometer flask contained in the vessel proper 1 ml of the same yeast suspension which had previously been treated in the boiling water bath for 30 minutes, and in the sidearm contained substrate, which was tipped in after a 10-minute equilibration period, exactly as with the experimental flasks. Any non-enzymatic decomposition of the substrate was thus controlled.

Substrate was prepared by dilution, in buffer, of reagent grade 30 per cent hydrogen peroxide. For those experiments involving extremely high peroxide concentrations, use was made of a small supply of 60 per cent H_2O_2 generously made available by Mr. C. J. Kellogg of the Buffalo Electro-chemical Company. In the section dealing with the effect of pH, substrate was unbuffered; the buffer mixtures there employed were McIlvaine's phosphate-citric acid in the range of pH 3–8, and glycine-NaOH for the range of pH 8.5–11.

Chloroform and hydrogen peroxide pretreatment.—In all experiments involving pretreatment of cells with chloroform before activity assay, 1 ml of the $CHCl_3$ was added to an Erlenmeyer flask containing 9 ml of the 1 per cent yeast suspension, which was more than sufficient to saturate the aqueous cell suspension; quantitative results on the effect of $CHCl_3$ concentration will be presented in the next paper in this series. This flask, and a control to which 1 ml of buffer was added, were placed on an air-driven rotary shaker (mounted on the temperature bath to be described below) and shaken for 30 minutes at approx. 70 strokes per minute over an arc of 5 inches; this was done at room temperature. The control and treated suspensions were then centrifuged and twice washed with buffer, and resuspended in buffer to the original 1 per cent suspensions which were then further diluted for activity assay. Not the slightest trace of enzyme was given up to the medium by the cell as a result of such treatment.

In the series of experiments describing the effect of pretreatment of the yeast with H_2O_2, aliquots of a one per cent suspension were introduced into Erlenmeyer flasks to which were then added equal volumes of the peroxide solutions to be tested, thus approximately reproducing conditions obtaining within the Warburg flasks. Values of peroxide concentration are expressed below as final concentrations after mixing. The flasks were then shaken at room temperature for 30 minutes, and allowed to stand in the cold for several hours until all trace of bubbling had ceased in the more concentrated solutions, since otherwise concentration of the cells by centrifugation

was difficult. The cells were then washed free of the peroxide, restored to the original concentration, and diluted with buffer for activity assay as above.

In some experiments to be described, chloroform pretreatment was followed by H_2O_2 pretreatment, in which case the procedures described above were simply combined consecutively. Some experiments were performed which will not be described below, in which hydrogen peroxide treatment preceded the chloroform treatment; here, the results were qualitatively like those of chloroform pre-treatment alone. In other words, H_2O_2 pretreatment did not alter the subsequent response of the cells to chloroform.

Heat pretreatment.—One-fiftieth per cent suspensions of cells were placed in Erlenmeyer flasks which were then clamped to the shaking device described above; experimental flasks were shaken in the constant temperature bath while controls were allowed to shake at the same rate and excursion outside the bath at room temperature. Aliquots were withdrawn periodically for viability and activity assay.

Ultraviolet pretreatment.—One-fiftieth per cent cell suspensions were placed in 4 inch petri plates for irradiation approx. 3 cm beneath the mercury vapor lamp described elsewhere (1, 24). The plate was placed on the stage of a superannuated microscope mounted in a snugly fitting, round holder which was turned at 1 rpm by a motor beneath. The stage was inclined at an angle of approx. 5° from the horizontal, and the lamp was tilted correspondingly to give maximum illumination; in this way the cells at the top of the plate were exposed continuously in a thin layer and a continuous, gentle stirring took place during the irradiation. Temperature changes greater than 0.5° C were not recorded beneath this quartz source. Control suspensions were run alternately; here, the glass petri dish covers were in place to shield the cells from the UV, but otherwise experimental conditions were the same.

Preparation of aqueous enzyme extracts.—Ten grams of wet cells were ground for 10 minutes in the cold room in a mortar to which 2 grams of Corning powdered pyrex (passes 20 mesh, retained on 40 mesh) had been added; 2 more grams of the pyrex were then added and the grinding continued for 10 minutes when this procedure was repeated once again, for a total of 30 minutes of grinding. The mixture was then extracted with 20 ml of buffer, and twice centrifuged and twice filtered. If the extract was to be used for more than 1 day, a drop of toluene was added as a preservative; while toluene is a potent activator of the intracellular enzyme, it was without action on the extracted catalase. Since no attempt was made to purify the enzyme further, this extract was really a solution of ribose nucleic acid contaminated with catalase and other soluble substances; this interpretation, confirmed by spectrophotometric analysis, does not restrain me from referring to this extract as a catalase solution, nor should it affect the results to be described.

In another series of experiments to which allusion is made below, a 10 per cent suspension of yeast was ground in the cold with a fixed amount of pyrex (one-half the weight of the yeast); at intervals, the mixture was stirred, and aliquots were withdrawn by means of a sawed-off pipette and were diluted, uncentrifuged, for activity and viability assay. These aliquots included cell debris and the increasingly fine powdered pyrex, as well as whole cells and the extracted, soluble material.

Presentation of data.—Since statistical treatment of these data was not feasible, Roman numerals have been placed on each figure to correspond to the number of experiments performed more or less exactly like the one in question with similar

results; Arabic numerals refer to the number of activity determinations averaged per point on the figure. Most of the effects to be described were first observed during the course of a year's preliminary research, which is not included on the figures to be presented. In some figures, the total range of scatter about the mean point is shown. Data which are only incidental to the main line of argument will be presented in small type.

Outline of experiments to be presented.—Preliminary experiments will show that activity of yeast catalase is directly proportional to cell and substrate concentrations; it will be seen that linearity of the oxygen evolution curves in no way indicates independence of substrate concentration.

Permeability of the cell membrane cannot explain the difference in 'optimum' substrate concentrations for catalase of the living cell and that extracted from the cell; the activity vs. substrate concentration curves for the extracted enzyme and for the $CHCl_3$-altered enzyme will be shown to be identical.

That the catalase of the intact cell is protected from inactivation by H_2O_2, relative to the extracted and $CHCl_3$-altered enzymes, will be shown by experiments in which the catalases are pretreated with their substrate before activity determination; differences in behaviour of normal and $CHCl_3$-altered enzymes cannot be due to catalase concentration.

The activity vs. pH curves of altered intracellular and extracted enzymes will be shown to be identical, both differing from the catalase of the intact cell. Effects due to the acidic properties of H_2O_2 are eliminated, and explanation of the pH-activity relationships in terms of cell permeability are seen to be unlikely, at least in the alkaline range.

Definitive proof of enzyme alteration is afforded by the demonstration of the greatly increased sensitivity to heat following alteration of the intracellular enzyme by $CHCl_3$.

UV radiation will be shown to produce qualitatively the same sort of alteration as does $CHCl_3$ with respect to the activity and heat sensitivity of intracellular catalase.

It should be noted that the above experiments were designed to demonstrate enzyme alteration, *not* as a test of the interfacial hypothesis.

THEORETICAL

Effect of enzyme concentration on substrate 'optimum'.—The brilliant work of Chance (for review see (7)) has clearly shown that the kinetics of enzymatic decomposition of hydrogen peroxide do not depend upon the rate of breakdown of the classical Michaelis enzyme-substrate complex (28). The reason for which the usual Michaelis treatment was apparently successful for so many years (i.e. permitting calculation of a Michaelis constant, for the catalase-H_2O_2 system) is that there does exist, as predicted by the theory, an 'optimal' substrate concentration, that is, a point at which increased substrate concentration does not lead to increase in activity. This fact has been known since the classical, but largely forgotten, studies of Lovatt Evans (27) and the later ones of Morgulis (29). Chance has now shown us that the

existence of the 'optimum' is not due to saturation of the enzymatic surface with substrate, but rather to inactivation of enzyme by the peroxide.

Lovatt Evans stated in his early paper that "the question which naturally suggested itself ... was whether the optimum point in the concentration of the H_2O_2 varied with the strength of the catalase solution, and if so, to what extent". If the 'optimum' were due to saturation of the enzymatic surface by substrate, as in the case of Michaelis-Menten treatment, the answer to Lovatt Evans' question should be 'no', since the per cent saturation of enzyme surface for a given substrate concentration should be independent of enzyme concentration. Actually Lovatt Evans found that this was not the case, since the 'optimal' concentration *decreased* with *lowered enzyme concentration*, not directly, but approximately as the square root of the latter. Qualitatively the same phenomenon was rediscovered exactly 40 years later by Bonnichsen, Chance and Theorell (5). That the explanation of this phenomenon lay in the inactivation of catalase by its substrate was likewise discovered by Lovatt Evans, who described 2 properties of catalase as "I., That there are optimum concentrations of hydrogen peroxide which are different for each new strength of enzyme employed. II., That when the relative amount of substrate is large, catalase is rapidly changed into an inactive form." The relevance of Lovatt Evans' work to my own will be made clear below.

Manometric methods. —One other aspect of my work should be considered in advance. Activity has been determined by means of the manometric technique described above, similar to that of Sizer (33). Now, any technique imposes certain limitations of enzyme and substrate concentrations, although this is rarely considered. In the case of the Warburg-Barcroft method, one must use enzyme and substrate concentrations on the one hand sufficiently dilute to permit several readings at at least 10 or 15 second intervals before the manometer fluid has reached its highest (or lowest) readable point, and on the other hand sufficiently high to permit the reaction to proceed rapidly enough to accommodate the exigency of an 8 hour working day. A linear curve is usually the result, but attempts to interpret this fortuity as a proof of zero order kinetics (33) must be regarded with suspicion, as will be shown.

Beers and Sizer (3) have recently cast doubt on the use of this technique. "The manometric method for measuring oxygen evolved from the system", they state, "proved in detailed studies to be unsuited for following the rapid breakdown of the peroxide in which a diffusion process across the liquid-air interface becomes limiting. This is manifested by changes in both the order of the reaction and the rate of evolution of oxygen with variations in the rate of agitation of the reaction mixture." Subsequent publication of these detailed studies (4) reveals that what actually changed with rate of shaking was the initial lag phase; their method of calculating their "zero order velocity constant" was to extrapolate the first point (or first 15 seconds) on their curves to the origin, rather than measure the slope of the linear portion of the curve! Since they state themselves that "The initial lag in oxygen evolution commonly observed has no theoretical basis in the catalytic process", it is rather surprising that they included this lag in calculations of "zero order velocity constant".

When their velocity constants are calculated properly from the slope of the linear portion of the curves rather than from the extrapolated initial rates, it is far from

sure that their data support their statement that the constants increase significantly with increased rate of shaking. That this unique method of calculating "zero order velocity constants" is incorrect is shown by their data relating catalase activity to substrate concentration: large increases in substrate concentration (even at concentrations of less than one-third the "saturation value" of the substrate) led to only minimal changes in activity, at all rates of shaking (see their Fig. 2). In any case, Mr. Fraser, of this laboratory, in an attempt to reproduce their data, has found that changing the shaking rate from 72 to 100 or 120 per minute leads to *no change whatever* in the oxygen evolution curves (including lag period) obtained by our methods. It will be seen below that activity measured from the slope of the linear portion of the curves is directly proportional to enzyme and to substrate concentrations; it is a bit hard to see how "a diffusion process across the liquid-air interface becomes limiting" under these circumstances. For these, and other, reasons, I feel that the contentions of Beers and Sizer do not apply to my work, despite their statements to the contrary (4).

However, there does exist one reasonable criticism of manometric methods in catalase assay, made over ten years ago by Theorell and Agner (37); they showed that specific activity measurements made manometrically are much lower than those obtained titrimetrically. This interesting and well-known (5) finding does not affect the present conclusions, since no evaluation of an absolute activity per molecule has been attempted. It might be noted that the original demonstration of the Euler effect was made by titrimetric methods (10). Furthermore, we shall show in a subsequent publication in this series (Fraser and Kaplan, in preparation) that turnover numbers for crystalline beef-liver catalase are no more than 25 per cent higher when determined titrimetrically than manometrically, and that energies of activation determined by the two methods are, within experimental error, the same.

RESULTS

Preliminary. The effect of concentration of yeast cells on enzyme activity is shown in Fig. 1, where 2 different suspensions were assayed at varying substrate concentrations; it will be seen that there is a constant ratio of activities of the two suspensions. In another series of experiments, the catalase activity of a 32-fold range of cell concentrations ($^1/_{32}$ per cent to 1 per cent) was examined at constant substrate concentration (4.4×10^{-2} M); the plot of activity vs. cell concentration was linear, and extrapolated directly to the origin, unlike the results of others (4). Within the range of substrate and enzyme concentrations used in the present experiments, activity is thus directly proportional to yeast concentration.

Fig. 2 represents two typical O_2 evolution curves whose slopes were used in the calculation of activity as there illustrated. Concentration of yeast was the same in both cases, but the substrate in curve *A* was 2.5 times the concentration of that in curve *B*, and the observed activities correspond fairly well to the difference in sub-

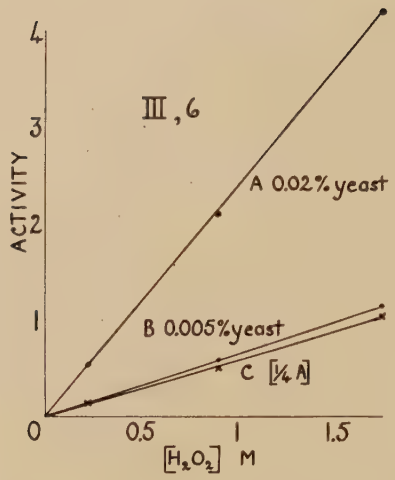

Fig. 1.

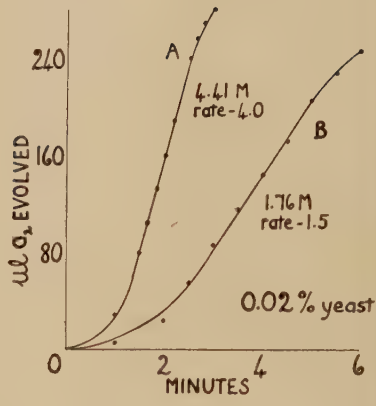

Fig. 2.

Fig. 1. Catalase activity of 2 yeast suspensions at varying concentrations of substrate. Curve *C* is theoretical ($^1/_4$ the slope of curve *A*).
Fig. 2. O_2 evolution curve of a 0.02 per cent yeast suspension at 2 concentrations of substrate.

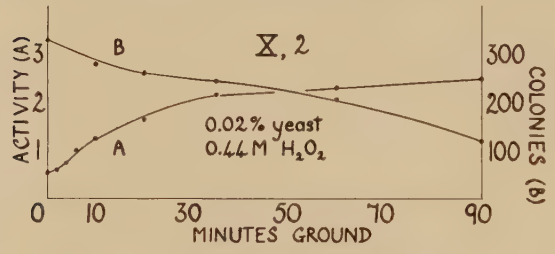

Fig. 3. The effect of grinding on catalase activity (*A*) and on viability (*B*) of yeast.

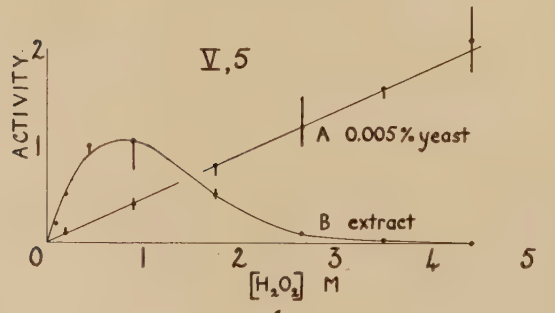

Fig. 4. Catalase activity of *A*, cells, and *B*, extract, at varying H_2O_2 concentrations. Total range of activities of which each point is the mean is shown.

strate concentration. Despite this difference in H_2O_2 concentration, it will be observed that, after the short lag period which persisted at high substrate concentrations unlike the results of others (4), both curves become quite linear; this linearity was the rule throughout these experiments *regardless of substrate concentration*. Linearity of these

TABLE I

Decrease in viability after 30 minutes' grinding; standard deviation shown.

Plate counts control cells	Plate counts after grinding
315±30	203±11

curves clearly cannot be taken to mean that the reaction rate is independent of substrate concentration; it is thus not possible on the basis of these curves to refer to catalase kinetics as being of zero order with respect to substrate concentration, as has been done by Sizer (33) and, alas, by myself (21). The linear curves, obtained over the entire range of substrate concentrations used in these experiments (2.2×10^{-2} M to 5.8 M), is apparently a fortuitous and convenient consequence of the manometric technique employed. It will also be noted from Fig. 2 that after the evolution of some 200 μl of O_2, the curve deviates from linearity, in the case of curve *A* at a point where less than 1 per cent of the substrate has been decomposed. Sizer (loc. cit.) observed this phenomenon at about the same level of O_2 evolution, although he used a concentration of H_2O_2 of 10^{-2} M, more dilute than any used here. Sizer attributed this to "some destruction of the enzyme by the peroxide"; this is probably not the correct explanation, as will be shown below.

Fig. 3 demonstrates that removal of the enzyme from the cell results in increase of its activity, much as has been described for the catalase of erythrocytes (20). Despite the continuous decline in viable cells, not much increase in activity takes place after 30 minutes' grinding, due, probably, to adsorption of the enzyme onto the constantly increasing surface of the powdered glass; that adsorption onto glass decreases activity of catalase has been shown by Rosenblum (31). When the technique of 30-minute grinding (described above) was employed, about one-third of the cells were destroyed, as shown in Table I; the aqueous extract thus prepared was used throughout the balance of the experiments to be described.

The possibility of a non-catalatic decomposition of peroxide, suggested by Grunberg-Manago *et al.* (18), which might conceivably account for the activity of cell suspensions at very high substrate concentrations, was ruled out by demonstrating the abolition of all activity when suspensions were tested in the presence of 10^{-3} M sodium azide.

Effect of substrate concentration.—In Fig. 4 is presented the variation in activity with substrate concentration of *A*, cells and *B*, extract. The cells exhibit a linear relation between activity and substrate concentration over the entire range, whereas the extract reaches a maximum between 0.44 and 0.88 M and is completely inactive at high concentrations. This interesting

finding that catalase is instantly inactivated at high concentrations of the H_2O_2 was previously reported by George (16) and later by Avi-dor and Yaniv (2). It will be observed that the intracellular enzyme is fully active at 4.41 M H_2O_2 while turning over substrate at twice the rate of the extract at the point where activity of the latter levels off (0.44–0.88 M); hence *permeability of the cell membrane* to peroxide cannot be invoked to explain the difference in behaviour of the enzyme in the two states. However, in view of the findings of Lovatt Evans cited above, it is possible that the observed difference in behaviour of the same enzyme before and after extraction from the cell may be due to the difference in the *concentration of the catalase*, on the one hand tightly packed within the cell, and on the other diffusely dissolved in an aqueous medium. This possibility has been ruled out by demonstration of the identical phenomenon under conditions where the enzyme is concentrated to the same extent; this was done by treating the enzyme *in situ* with chloroform (see below).

Attempts to verify the results of Lovatt Evans were not successful, probably due to limitations of the manometric technique employed. A range of dilutions of enzyme extract was run at various peroxide concentrations. The practical upper limit of enzyme concentration is that at which 200 μl of oxygen are given off in approx. 30 seconds; although this (1:25) extract is some 30 times as concentrated as that normally used in these experiments, its activity is less at 1.76 M than at 0.88 M (as is the case with the most dilute extract), and it possesses comparatively little activity at 4.41 M during the first 60 seconds of the run, becoming rapidly inactive thereafter even though only approx. 120 μl of O_2 had been set free. A typical experiment is shown in Table II; it should be emphasized that the activities of the more concentrated enzyme extracts were only rough guesses since the reactions were too fast to permit accurate readings.

Evidence that the *time* at which activity is assayed is important in determining the existence of an 'optimum' substrate concentration is presented in Fig. 5, where an extract is run at different concentrations of peroxide. In Curve *C*, 1.76 M H_2O_2, it will be noted that although the enzyme possesses considerable activity during the first two minutes, it becomes completely inactive at 4 minutes. That an extract possesses considerable activity at 1.76 M H_2O_2 (Fig. 4 *B*) is clearly due to the fact that activity was determined as the slope of the curve obtained from the first 1 to 2 minutes of the run, before complete inactivation had occurred. In this intermediate range of substrate concentrations (0.88 M–2.64 M), the observed activities of the extract are obviously partly determined by the extent to which catalase has been inactivated by its substrate. The observations contained in Table II and Fig. 5 *C* suggest the importance of *time of measurement* in determining the existence or location of an 'optimal' substrate concentration. The *temperature* of measurement also seems to play a part in location of the substrate 'optimum'; the statement of Beers and Sizer (loc. cit.) that the substrate 'optimum' changes with rate of shaking of the reaction vessels is contradicted by their own data (see their Fig. 2).

TABLE II

Effect of dilution of an extract on activity at 2 different concentrations of substrate (II, 3).

Dilution	Activity		Remarks
	0.44 M H_2O_2	4.41 M H_2O_2	
1:25	too fast	2.1	At 0.44 M reaction too fast to estimate. At 4.41 M complete inactivation occurs at approx. 60 seconds.
1:50	13	0.02	Only very slight initial activity at 4.41 M; rapid inactivation thereafter.
1:100	9	0	
1:200	4	0	
1:400	2	0	

A further argument against the permeability hypothesis is presented in Fig. 6, in which the activity of yeast cells at very high substrate concentrations is shown. Beyond a point of maximum activity at approx. 6 M, their activity is reduced, and at 8.8 M has been completely eliminated: at this substrate concentration *the cell suspensions behave exactly like thermobarometers.*[1] Hence, the cell membrane must be freely permeable to H_2O_2 in order for an instantaneous and complete suppression of activity of the intracellular catalase to occur.

The effect of chloroform pretreatment on the substrate-activity curve of the whole cells is shown in Fig. 7. It will be noted that the general shape of the curve has been shifted from that shown in Fig. 4 *A* to that of Figs. 4 *B*, despite the fact that the concentration of enzyme is the same in both Fig. 4 *A* and 7. The balance of the data to be presented in this paper are adduced to show that the reason for the resemblance between intracellular chloroform-treated catalase and the extracted catalase is that the chloroform has transformed the intracellular catalase into a condition qualitatively like that which it possesses upon extraction from the cell; in other words, to show that both extraction and chloroform treatment alter the enzyme in such a way as to transform many of its properties. Treatment of the 1 per cent suspension with chloroform resulted in almost complete sterilization of the population of yeast.

[1] Extracts, prepared by grinding cell suspensions which had been in contact with 8.8 M H_2O_2, were found to have no catalase activity whatever, showing that high concentrations of substrate inactivate instantly the intracellular enzyme itself, rather than act on the cell membrane in some way.

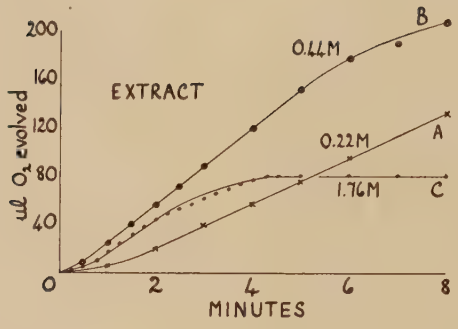

Fig. 5. O_2 evolution curves of an extract run at different concentrations of substrate.

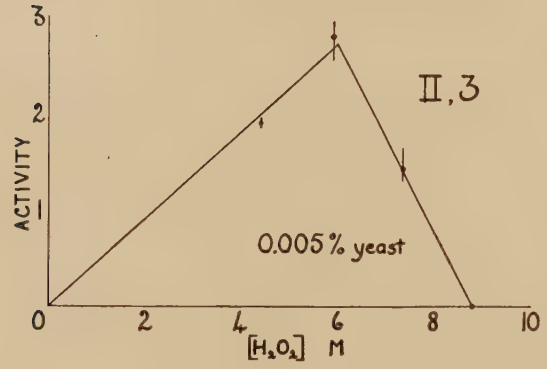

Fig. 6. Catalase activity of cells at very high concentrations of substrate. Total range shown.

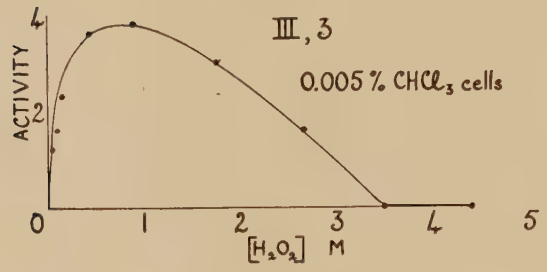

Fig. 7. Effect of $CHCl_3$ pretreatment on the catalase activity of cells at varying concentrations of substrate.

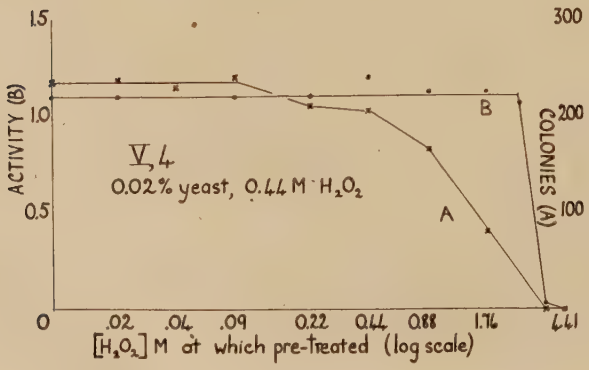

Fig. 8. Effect of H_2O_2 pretreatment on *A*, catalase activity, and *B*, viability of cells. Semi-log scale used simply for convenience in drawing graph; final molar concentrations of H_2O_2 used in of the pretreatment are shown.

Comparison of Fig. 4 *A* and 7 illustrates the very great increase in activity following pretreatment of intracellular catalase with chloroform, which is the Euler effect. Chloroform treatment of the extracted enzyme usually did not affect its activity, but occasionally a *decrease* in activity was noticed. This irregularly observed decrease was possibly due to adsorption of the enzyme onto droplets of the chloroform emulsified by shaking.

Pretreatment with hydrogen peroxide.—The effect of prolonged treatment with H_2O_2 on the activity and viability of yeast cells is shown in Fig. 8. Curve *B* shows that no catalase destruction whatever took place until the enzyme had been pretreated with high concentrations of the order of 3.5 M, where irreversible destruction did occur. Curve *A* shows that the cells were rather well protected by their catalase, at least up to a certain point, since not all were killed until all their catalase had been inactivated. Yet even at these very high peroxide concentrations the catalase was initially active (see Fig. 4 *A*), and the work of George (16) and Avi-dor and Yaniv (2) suggests that dilution of the cells after a reasonably short contact with substrate of this concentration would have protected the enzyme from any irreversible inactivation.

Fig. 9 *A* shows the effect of a half-hour pretreatment with H_2O_2 on a concentrated extract, which was then diluted for activity determinations. Again, no destruction of enzyme whatever took place after treatment with substrate of up to 0.44 M, but some irreversible loss of activity occurred with 0.88 M, and total destruction between 1.8 and 3.5 M.

Since the 'tapering off' of the O_2 evolution-time curves is the same for extracts at concentrations of substrate between 0.02 M and 0.44 M as described above in the case of intact cells, one may conclude that this deviation from linearity does not involve 'destruction' of the enzyme; it may be an unexpected consequence of the manometric technique, since it usually occurs at about the same level of evolution of gas into the Warburg vessel, or it might be a temporary inhibition. Two separate experiments were run on a sample of crystalline beef liver catalase for purposes of comparison; here also, no decrease in activity occurred after treatment at H_2O_2 concentrations of less than 0.22 M, but some inactivation was noted after treatment with 0.22 M peroxide, and all activity was lost after 0.88 M.

Fig. 9 *B* shows the effect of peroxide pretreatment of a population of cells which had previously been chloroformed. It will be noted that the results are qualitatively similar to those obtained by pretreatment of the extract. Chloroform pretreatment as well as extraction of the enzyme thus render the enzyme sensitive to concentrations of peroxide (0.88 M–2.64 M) to which the enzyme within the untreated cell is indifferent. It might be well to repeat

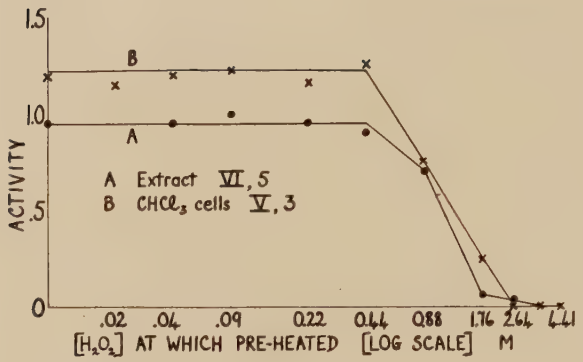

Fig. 9. Effect of H_2O_2 pretreatment on catalase activity of *A*, extract, and *B*, $CHCl_3$ pretreated cells. X-axis should read: (H_2O_2) at which pre-treated.

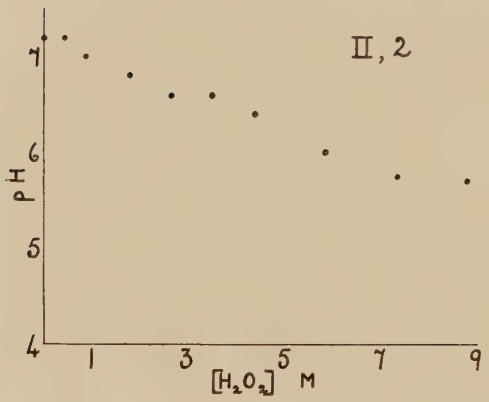

Fig. 10. Effect of H_2O_2 on the pH of the phosphate buffer. Equal volumes of the peroxide solutions and the phosphate buffer (pH 7.2) in which cells were suspended were mixed and pH determined.

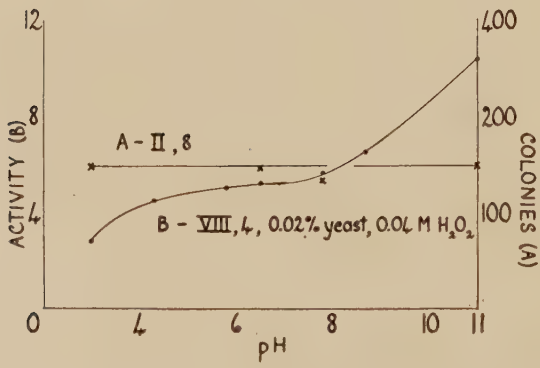

Fig. 11. Effect of pH on *A*, viability, and *B*, catalase activity of cells.

that no trace of catalase whatever is liberated into the medium from the cells as a result of chemical pretreatment. Since the concentration of enzyme within treated and untreated cells is the same, the difference both in absolute activity and in sensitivity to substrate must be due to some other factor.

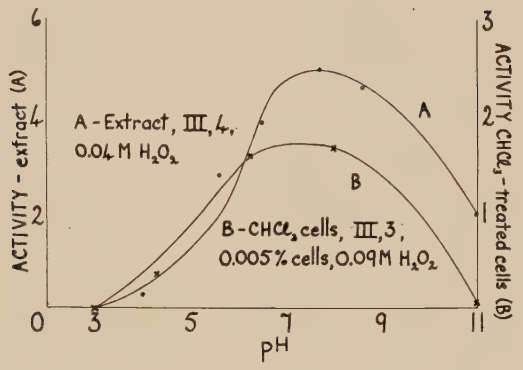

Fig. 12. Effect of pH on the catalase activity of *A*, extract, and *B*, $CHCl_3$ pretreated cells.

That this factor might be permeability of the cell has already been shown to be unlikely. That this factor is enzyme alteration, that is, a change in some property of the enzyme itself, rather than some extrinsic factor, will now be demonstrated.

The effect of pH.—It is well known that hydrogen peroxide dissociates as a weak acid (9); it thus seemed possible that the inactivity of catalase at high substrate concentrations might be due to an increased hydrogen ion concentration. Fig. 10 shows the pH of the various reaction mixtures of the previous experiments; pH values were determined electrometrically, in the absence of yeast. It will be remarked that at the highest H_2O_2 concentration employed, the pH of the buffer had been lowered to 5.7.

Fig. 11 shows the effect of diluting the washed cells in buffer mixtures of varying pH for activity assay. Curve *A* shows that the viability was not affected, and curve *B* relates activity to pH. The activity of the intracellular catalase was somewhat reduced at low pH, 3.0–4.3, reached a fairly constant level between pH 5.8 and 8, and showed a definite increase at extremely alkaline pH, 10 to 11.

Fig. 12 represents changes in activity at varying pH of the extracted enzyme, *A*, and the chloroform pretreated cells, *B*. Both were qualitatively quite similar, being completely devoid of activity at pH 3, and of greatly reduced activity at pH 11. They possessed considerable activity at pH 5.5, hence the acidic properties of H_2O_2 cannot be used to explain the inactivation of catalase at high substrate concentrations. It should be emphasized that all of these experiments on pH have been run using concentrations of substrate of an order of magnitude below the 'optimal' level for the extracted, and chloroform-treated, enzymes; hence, if the action of alkali has simply been to increase the permeability of the cell to substrate, the activity of the chloro-

formed cells ought likewise to have increased at high pH. On the other hand, it is evident that some of the alkaline ions have penetrated the living cells since a marked increase in catalase activity was noted; if a weakly dissociated (hence more freely penetrating) base, such as ammonium hydroxide, was used, a much greater activation was noted at pH 11.

The interpretation of these curves is indeed complicated, but it is evident that the difference in the alkaline range between control and chloroformed cells can be attributed neither to permeability of the cell membrane nor to concentration of the enzyme, unless one is willing to make a series of quite complicated assumptions. The enhanced sensitivity of the catalase to high hydrogen ion concentration following $CHCl_3$ treatment of the cell was noted by Euler and Blix (10). This difference may largely be due either to low permeability of the intact cell to hydrogen ions, or to the buffering capacity of its protoplasm, or to both. However, I have some evidence, not to be presented here, suggesting that at least part of the difference is due to change in sensitivity to hydrogen ion of the enzyme itself following $CHCl_3$ treatment of, or extraction of the catalase from, the cell.

The effect of heat.—I have sought definitive evidence for enzyme alteration that would rule out clearly and unequivocally hypothetical activators, inhibitors, permeability of the cell, and concentration or amount of the enzyme; while presumptive evidence of enzyme alteration has been presented above, the definite demonstration of the validity of this hypothesis must stand or fall on evidence presented in this, and in the succeeding, section. My previous work had shown that extracted red cell catalase was relatively sensitive to heat as compared to the intracellular enzyme (20). Fig. 13 shows the course of change in activity and viability as untreated and chloroformed cells were shaken side by side at 56° C. Curve *A*, the untreated suspension, shows initially the same increase in activity (heat activation) reported in the case of erythrocyte catalase; after one hour, the activity began to decrease below that of the zero hour (unheated) aliquots, and total destruction of the enzyme took place after 3 hours. Curve *B*, activity of the chloroformed cells, shows initially a very high activity (Euler effect), but then demonstrates almost total loss of activity after but 15 minutes at 56°, whereas the control cells required 3 hours. Curve *C*, viability of the unaltered yeast, demonstrates almost complete sterilization of the population after 15 minutes at this temperature.

In order to compare these cells with the erythrocytes previously used, four additional experiments were run at 50° C. In this series, the activity of the untreated yeast cells remained, upon heating, higher than that of the

unheated (zero hour) cells for approx. 5 to 6 hours, and possessed slightly more activity than the thermobarometer even after 13 hours at this temperature. The chloroformed cells, on the other hand, had lost all trace of activity between 2 to 4 hours at 50° C.

In another series of experiments, chloroformed and untreated 1 per cent cell suspensions were heated at 56° C for 1 hour, when their activities were determined; as before, the untreated cells were active, whereas the chloroformed cells had lost all trace of catalase. Then, extracts of these heated suspensions were prepared in the usual way, and their activities were determined. Prior to heating, an extract from the untreated cells had been made, and this extract, originally highly active, had been heated alongside the 2 cell suspensions; its activity could then also be determined after 1 hour at 56° C. These data are summarized in Table III; they prove that heat has abolished the activity of the extracted catalase, and has rendered inactive the extract subsequently prepared from the chloroformed cells. The extract, prepared from the untreated cells which had been heated for the same duration at the same temperature, possessed high activity even after 50-fold dilution. This experiment, demonstrating presence or absence of enzymatic activity in extracts after a standard period of heating of the control and chloroformed cells, and of an initially active extract itself, can be explained only by an alteration of enzymatic character following chloroform treatment of the intracellular enzyme or following its extraction from the cell; this alteration has increased the activity of the catalase, but has rendered it sensitive to the action of heat.

The action of UV.—The effect of UV is quite different from heat but much more interesting, as will be seen in Fig. 14. Curve *B*, activity of the chloroformed cells, shows a more or less regular decrease with time from its initial, very high value. Curve *C*, the irradiated extract from untreated cells, is

TABLE III

Demonstration of presence or absence of enzymatic activity in catalase extracts after 1 hour at 56° C (II, 3).

	Activity
A. Control cell suspension, heated	0.82
B. Extract prepared from *A* (extract itself unheated)	2.22
C. Chloroformed cell suspension, heated	0
D. Extract prepared from *C* (extract itself unheated)	0
E. Extract prepared from unheated control cell suspension (extract itself heated)	0

A and *B* diluted 1:50 before activity determination. *C*, *D*, and *E* were undiluted.

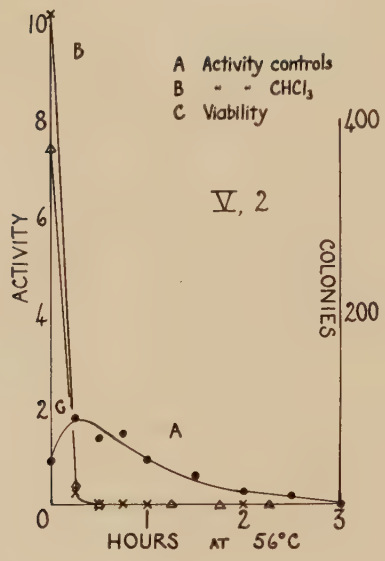

Fig. 13.

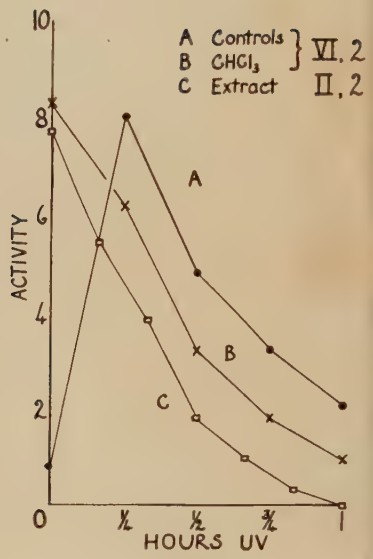

Fig. 14.

Fig. 13. Effect of heat (56° C) on the catalase activity of *A*, cells, and *B*, $CHCl_3$ pretreated cells, and on *C*, viability of cells.
Fig. 14. Effect of UV irradiation on the catalase activity of *A*, cells, *B*, $CHCl_3$ pretreated cells, and *C*, extract.

quite similar in character to Curve *B*. Curve *A*, activity of the untreated suspension, shows a dramatic increase in activity during the first 15 minutes of the irradiation, reaching the level of activity of the unirradiated, chloroformed cells; this great activation by UV was discovered by Euler and Guenther (12) and by Fuller (14). However, after the UV activation, the activity of these cells falls off at approximately the same rate observed in curves *B* and *C*; after 15 minutes, the population is unable to form colonies.

The obvious explanation of curve *A* is that the intracellular enzyme is altered by some consequence of the action of UV on the cell to a condition like that which it possesses after chloroform treatment; in other words, UV causes enzyme alteration, as does chloroform, thus enormously increasing the catalase activity, but rendering it sensitive to the continuing irradiation. Heat, on the other hand, does not cause enzyme alteration.

This is quite easily proven, since if so, untreated cells which have been first heated should not change in their subsequent response to UV, whereas cells which have first been irradiated should behave like the chloroformed cells with respect to subsequent heat. That this is indeed the case is shown

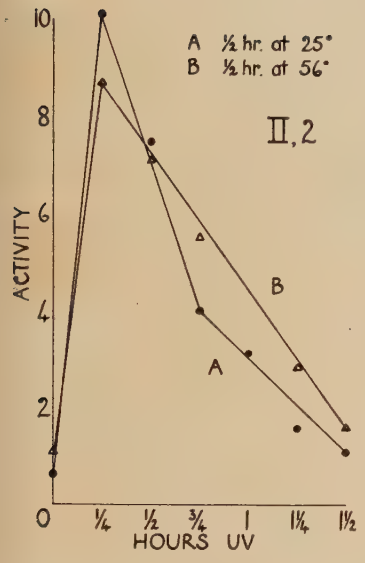

Fig. 15.

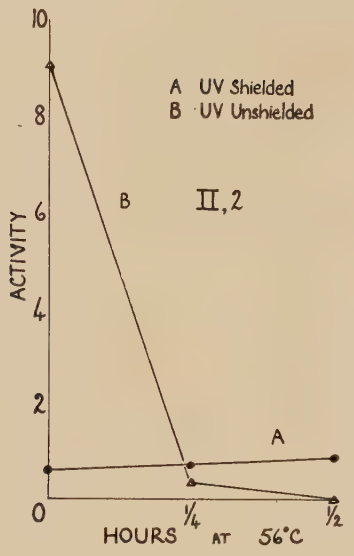

Fig. 16.

Fig. 15. Effect of UV on the catalase activity of *A*, cells maintained for one-half hour at 25° C, and *B*, cells maintained for one-half hour at 56° C.
Fig. 16. Effect of heat (56° C) on catalase activity of *A*, cells irradiated with glass cover as shield, and *B*, cells irradiated unshielded (15 minutes).

in Figs. 15 and 16. In Fig. 15, two untreated cell suspensions were shaken for one-half hour at 25° C (*A*) and 56° (*B*), following which they were both irradiated before activity was determined. The heated suspension, *B*, although initially somewhat higher in activity (compare Fig. 13 *A*), was capable of undergoing the same very large activation as was the unheated suspension, *A*. In Fig. 16, the effect of UV followed by heat is illustrated: Curve *A*, the activity of the control suspension which had been shielded from the UV by a glass cover, is virtually the same as the initial section of 13 *A*; a slight increase in its activity as a result of heat is noted, as above. Curve *B* represents the change in activity of the catalase of the previously irradiated (15 minutes) suspension: it is exactly like the change observed following heating of a suspension of chloroformed cells, shown in Fig. 13 *C*, that is, a rapid disappearance of activity after but 15 minutes at 56°.

These experiments clearly show that UV produces the same sort of alteration of catalase as does chloroform and extraction, and are irreconcilable with any hypothesis not involving some change in the properties of the enzyme itself, that is, enzyme alteration.

DISCUSSION

The nature of the Euler effect.—When chloroform was used to achieve the Euler effect (that is, the approximately 15-fold increase in catalase activity of a yeast suspension), certain other properties of the intracellular enzyme were also changed. The catalase within the $CHCl_3$-treated cells differed from the same enzyme within the living cells in the following respects: (i) its 'optimal' substrate concentration was lowered, due to (ii) its increased sensitivity to inactivation by H_2O_2; (iii) the pH at which its activity is maximal was changed; (iv) heat at a given temperature caused decrease in its activity at a faster rate and abolished activity after a much briefer duration of exposure; (v) UV was no longer capable of activating further the intracellular catalase. Catalase extracted from the cell has also acquired enhanced activity, and differs from the original intracellular enzyme in the 5 points above, sharing with the $CHCl_3$-treated enzyme the same optimal substrate concentration, the same concentration of H_2O_2, pretreatment with which is required to diminish residual activity, the same pH characteristics, the same response to heat and to UV. These qualitative and quantitative similarities in behavior indicate that chloroform pretreatment has changed the intracellular catalase to the same condition which it possesses upon extraction from the cell.

That permeability of the membrane of the intact cell may be a factor in the Euler effect seems unlikely, since the unaltered intracellular enzyme is instantaneously and completely inactivated by sufficiently high concentration of substrate. Furthermore Euler (10) showed that the Euler effect was not accompanied by any change in invertase activity, thus ruling out a general increase in cell permeability. In any case, the concomitant changes in properties, especially the effect of heat, cannot be explained by supposing changes in cell permeability. Similarly, whereas inhibitors, activators, or an actual increase in amount of enzyme might explain the Euler effect, they cannot explain the changed properties accompanying the increase in activity. The only explanation consistent with all these data is that the catalase itself has undergone a change in properties, resulting from treatment of the cell with $CHCl_3$ or UV, or from extraction of the enzyme from the cell: that is, enzyme alteration has occurred. Kinetic data from studies currently in progress strongly support this interpretation.

Generality of enzyme alteration.—There is evidence that enzyme alteration can occur in other cell types and with other enzymes. The interesting work of Herbert and Pinsent (19) on bacterial catalase is a case in point. They

found that, after lysis of *M. lysodeikticus* suspensions by lysozyme, the catalase activity increased 10-fold. They state that "the simplest explanation is that diffusion of substrate is a limiting factor". It seems, in view of the results of the present paper, that enzyme alteration may well have occurred in this case. These authors have used the extracted enzyme as a model of the intracellular catalase in calculating from the kat. f. values of the purified extract the number of catalase molecules per cell, and in concluding that catalase accounted for 1–2 per cent of the total dry weight of this bacterium. They state, "this is about ten times the catalase content of human red blood corpuscles". The tacit (though not unreasonable, lacking information to the contrary) assumption has been made that the enzyme possesses identical activity inside and outside of the cell. In my own work on cattle red cell catalase, I found that the activity of this enzyme doubled upon extraction from the cell. Assuming that human and cattle red cell catalases behave in the same way upon extraction, and further, that enzyme alteration has occurred in the case of the *Micrococcus* catalase as with the yeast and red cell enzymes, it would appear that the bacterial catalase would represent on a dry weight basis only about twice the catalase content of erythrocytes. In any case, in view of the data presented in the present paper, it would seem well-advised to bear in mind the possibility that the specific activity of an enzyme does depend on its physico-chemical configuration and this might change upon extraction from the cell.

In microbiology, there has been an increasing tendency to compare the enzymatic activity of whole cell suspensions with that of cell-free extracts or that of lysed cells (25, 26, 30, 35). In all of the cited papers, and in many others which could be mentioned, there was observed either a great increase in some enzymatic activity following destruction of cellular integrity or the appearance of activity not detected in the whole cells. Usually permeability effects have been held responsible, but the possibility might be considered that in some of these cases an actual enzyme alteration has occurred; experimental test of such an hypothesis is not difficult, as has been shown in the data presented above.

Alteration of catalase in plant cells is suggested by the increase in activity which follows their UV treatment, as reported by Fuller (14) and Frenkel (15), and in sea urchin sperm by the increase in catalase activity noted when determinations are made in distilled water (32).

One of the most striking parallels to certain of the present data is the discovery of Bovie that non-lethal doses of UV greatly sensitize paramecia to subsequent heat treatment (for literature, see (17)). Giese states in his

review (17) "The paramecia were irradiated with a fraction of a lethal dose of UV and then exposed to heat of 42° C. for less than the time which would normally kill by heat alone. The data indicate that even small fractions of a lethal UV dose sensitized the animals greatly to heat." Prior heating, on the other hand, did not sensitize the animals to subsequent UV. The interpretation of this striking phenomenon in terms of an UV-induced alteration of 1 or more enzymes which are, like catalase, resistant to heat before the irradiation, seems quite feasible.

Finally, it might be noted that Euler and Blix (10) reported that phenol pretreatment activated yeast fermentation, carboxylase and catalase; toluene and chloroform pretreatment seemed to 'provoke' phosphatase activity not previously present.

Significance of enzyme alteration.—The demonstration of this phenomenon in an enzyme which is evidently not associated with any of the formed elements of the cell (8, 34) seems to me to necessitate the re-introduction, in modified form, of the concept of the 'organized ferment'. Buchner's discovery (6) of the fermentative capacity of cell-free yeast juice has led to the accepted view of today, expressed by Sumner and Myrbäck (36), that "from an enzymological point of view, there is no difference between organized and unorganized ferments". This view must be re-examined in light of the present experiments showing changes in the enzymological character of catalase following its extraction from the cell, or following its alteration within the cell.

This revised concept would hold that an intracellular enzyme might possess an organization, or configuration, within the cell unlike that which it possesses upon extraction, and that as a consequence of this difference, enzymological properties might likewise undergo change. Re-introduction of the "organized ferment" hypothesis might cause workers in this field to become more aware of the possibility that some soluble, extracted enzymes might be highly inaccurate models of the same ferments within the cell, and might stimulate research leading to discovery of model systems exhibiting behavior more closely resembling that of intracellular enzymes.

Prospectus.—While the subject of the *mechanism* of enzyme alteration is to be treated in the next paper in this series, brief mention should be made of the types of hypothesis available. In addition to the interfacial hypothesis, other possibilities include: the cleavage of a catalase molecule of higher molecular weight into more active fragments; the splitting of a complex between catalase and lipid or protein constituents of the protoplasm; the liberation of the catalase from a loose combination with some cellular

structure (a possibility implied in (11)); the freeing of the catalase from a complex with an inorganic ion which previously had stabilized it against inactivation by heat. Doubtless other hypotheses might be constructed, and, indeed, may well become necessary. In the next paper, evidence will be adduced to show that some interfacial process must be involved directly or indirectly in enzyme alteration, since a striking correlation has been shown to exist between the ability of agents within or among certain homologous chemical series of compounds to cause enzyme alteration and to reduce the energy at a water surface.

SUMMARY AND CONCLUSIONS

A. The increase in catalase activity following treatment of yeast cells with chloroform (the Euler effect) has been accompanied by changes in many of its other properties. These changes include: decrease in 'optimal' substrate concentration, due to increased sensitivity to H_2O_2; altered pH-activity relationships; great increase in sensitivity to heat; changed response to UV irradiation.

B. These changes, both qualitative and, in so far as studied, quantitative, also accompany extraction of the catalase from the cell.

C. The only explanation consistent with these facts is that $CHCl_3$ treatment, or extraction, have caused *enzyme alteration*, that is, a change in the properties of the catalase itself. The properties of the catalase either altered by extraction from the cell or altered *in situ* with chloroform are identical.

D. The activation of intracellular catalase following UV irradiation, also discovered by Euler, has been shown to be due to enzyme alteration.

E. The phenomenon of enzyme alteration is believed to be restricted neither to yeast nor to catalase, and hence may necessitate the re-introduction of a revised concept of the 'organized ferment'.

Valuable assistance in design and construction of apparatus has been given by Mr. George Sandoz of Halifax; technical assistance has been rendered by Miss Marie T. Kennedy of St. Johns, Newfoundland. Certain extended conversations with Dr. Frantz A. Vandenheuval of the Atlantic Fisheries Experimental Station, Halifax, with Dr. Piotr Slonimsky of the Laboratoire de Génétique, University of Paris, and with Dr. Jacques Monod and his collaborators at the Institut Pasteur, Paris, were of great help. Finally, I must acknowledge my great debt to my colleague, Professor John Gray Aldous, Department of Pharmacology, Dalhousie University, whose point of view (1) is in many respects similar to my own, and whose time, techniques and equipment he has allowed me to exploit quite shamelessly.

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THE ANISOTROPIC AND ISOTROPIC BANDS OF SKELETAL MUSCLE IN LIGHT- AND ELECTRON MICROSCOPY

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Received June 21, 1954

In view of the recent evidence as to the distribution of actin and myosin in the anisotropic and isotropic bands of the striated fibril (3, 6) an exact determination of their length under different conditions seemed of renewed interest. A systematic investigation has hitherto been performed only by means of light microscopy on living fibres (1, 2). However, due to the fact that the width of these bands is near the resolving power of the microscope with ordinary light, and due to the complex optical conditions which arise from the fine striations and the cylindrical shape of the living fibre; such measurements are subject to an inevitable degree of inaccuracy. A. F. Huxley and Niedergerke (4) tried to avoid these artefacts by using an interference microscope and found contrary to findings with the ordinary light microscope, that the changes with passive stretch took place almost entirely in the I-band of the fibre.

A different approach has been used in the present investigation. Optical artefacts are avoided in electron microscopy. This procedure requires, however, fixed material. Therefore, in the present study we have compared findings on fixed material by light microscopy with findings by electron microscopy. Inasmuch as these were the same, it could be concluded that findings by light microscopy on living fibres are valid, since optical artefacts are the same for living and for fixed fibres.

METHOD

Single fibres were isolated both from living and from fixed semitendinosus muscles of *Rana esculenta* and *Rana temporaria*. The living fibre was photographed at different degrees of stretch from equilibrium length to a stretch of about 60 per cent, using a polarizing microscope with crossed Nicols (magnification 225 times).

To obtain fixed fibres in a non-contracted state at different degrees of stretch, the whole muscle had to be fixed. It was strongly curarized (0.5 mg tubocurarine chloride per gram frog) and placed in an isometric torsion myograph. Before fixation, the muscle was allowed to adjust fully to its imposed length for two hours in Ringer's

solution containing 50 μg per ml tubocurarine chloride at a pH of 7.2 and a temperature of 2 or 20 degrees centigrade. In order to avoid contraction and to minimize structural changes produced by the fixating agent, an amount of 40 per cent neutral formalin was added by an automatic syringe in the course of one hour to bring the Ringer's solution to a final formalin concentration of 2 per cent. The fixation time was 40–45 hours.

For light microscopy, fibres were isolated from the fixed muscle in distilled water and photographed in polarized light. From each microphotograph, the length of at least ten successive A and I bands was measured with a measuring microscope at a total magnification of 2500 times.

Fibrils for electron microscopy were obtained by fractionating the fixed muscle in a Waring Blendor in 150 ml of distilled water for 30 minutes. Blending was interrupted every three minutes to allow cooling by immersing the container in ice water. A platinum loop droplet of the fibril suspension was placed on a Formvar membrane on the metal grid used as specimen support for electron microscopy. The preparation was allowed to dry in air at room temperature.

The electron microscope was an R.C.A. type EMU 2B. The fibrils were microphotographed at a magnification of 3600 times. The magnification was calibrated by means of a metal replica of an optical grating.

The negatives of the electron micrographs were measured under the measuring microscope at a total magnification of about 35,000 times. Only the sarcomeres near the center of the field were measured to avoid errors due to distorsion near the edge of the micrographs.

To compare fibrils obtained by fractionating in the Waring Blendor with those from sections of the muscle, a piece of the fixed muscle was embedded in plastic, according to the method of Newman, Borysko and Swerdlow (8). Thin sections were obtained by using a Spencer microtome with a glass knife as described by Latta and Hartmann (7).

RESULTS

Preparation artefacts: Although no contraction of the muscle occurred during the addition of formalin to the Ringer bath, a slow increase in tension usually did occur about one hour later and continued for about three hours. The final increase for nine different muscles was 100–600 mg corresponding to about one to six per cent of P_0.[1] No further change occurred within the fixation period. The length of the sarcomere (A plus I substance) remained unaltered as evidenced by microphotographs of the muscle suspended in Ringer's solution before and after fixation. Hence, with the muscle fixed isometrically, the increase in tension must originate from shrinkage of one and equivalent stretch of another part of the sarcomere.

Strong mechanical stress to which a fibre is subjected during preparation,

[1] P_0 = maximum isometric tetanic tension, or resting tension at the indifference point where the stretched muscle no longer responds to stimulation with an increase in tension.

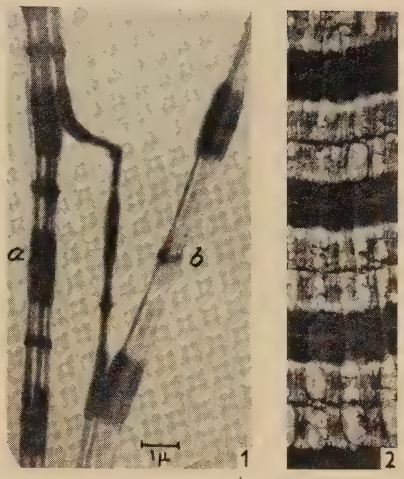

Fig. 1. *Electron micrographs of:* 1. Bundle of myofibrils at 90 per cent stretch (a), with one fibril split off at a stretch of about 250 per cent (b). I band elongated by 2.5 times, A band unchanged as compared with (a). (Sectioned preparation, fixed in 4 per cent formalin Ringer). — 2. Shrinkage of the A band and elongation of the I band in strong fixative (Sera's solution: 60 parts ethanol, 30 parts 40 per cent formalin and 10 parts acetic acid). The I band reached its breaking length and is partly ruptured at its junction with the A band. (Sectioned preparation.)

or fixation in strong fixatives, indicated that the A band in a fixed muscle has a higher stiffness than the I band. This is illustrated in the electron micrograph (Photo 1, Fig. 1) of a sectioned preparation stretched 90 per cent (a). One fibril is split off (b) and stretched by about 250 per cent of the equilibrium length. In spite of the large increase in the length of the sarcomere, the length of A is the same as at 90 per cent stretch, while the I band is stretched to 2.5 times its length at 90 per cent stretch. In Photo 2 (Fig. 1), the muscle has been subjected to a strong fixative. This results in so large an increase in tension due to shrinkage that the I band is ruptured at its border, while the length of the A band is only about half its value before fixation. Thus it is seen that in the fixed fibre the A band has a much higher stiffness than the I band, the ratio of per cent stretch of A : per cent stretch of I being as small as 1 : 6 (see page 332).

Fractionation in a Waring Blendor as such was seen not to induce a change in the length of the sarcomere. In the same fixed muscle, the mean length of the sarcomere was 3.42 $\mu \pm 0.025$ μ in isolated fibres; 3.31 $\mu \pm$ 0.035 μ in Waring blended fibrils—a difference of 3.2 per cent.

Drying the muscle fibrils on the Formvar membrane decreased the length of the sacrcomere by three to fifteen per cent. Comparisons of the A and I bands were therefore made at equal length of the sarcomere.

Plastic embedding and sectioning introduce additional shrinkage, the ratio of per cent stretch of A : per cent stretch of I being changed as compared with the ratio in the Waring blended fibrils. At what step of the procedure this shrinkage occurs needs further investigation.

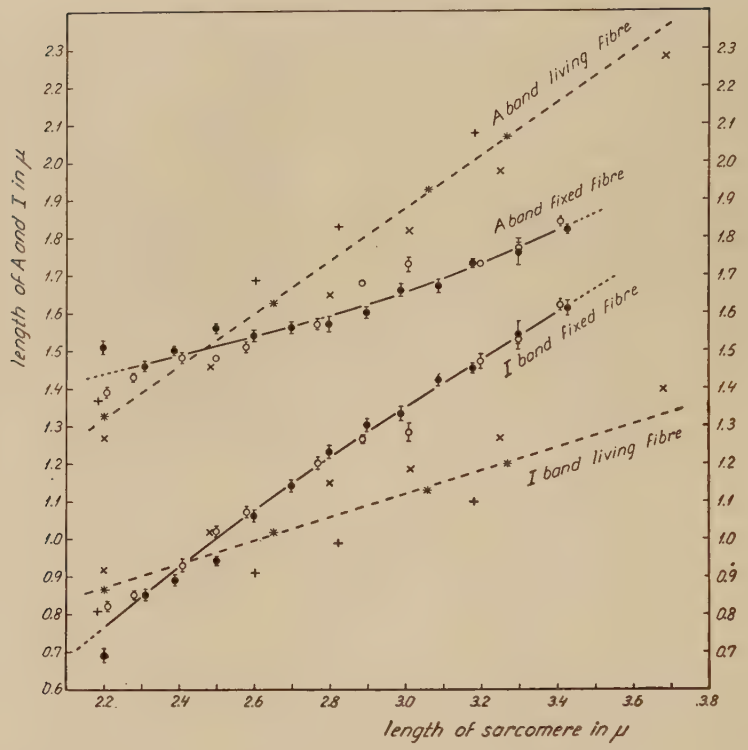

Fig. 2. Length of the anisotropic and isotropic bands as a function of stretch. Abscissa: length of the sarcomere in μ. Ordinate: length of the A and I band in μ.

Living fibres { + — + — + A and I bands in ordinary light (2).
⁎ — ⁎ — ⁎ A and I bands in ordinary light (1).
× — × — × A and I bands in polarized light (present study).

Fixed fibres and myofibrils { • — • — • A and I bands, electron microscopy.
○ — ○ — ○ A and I bands, microphotography in polarized light.

The vertical lines indicate mean error.

Length of the A and I bands in living and fixed muscle fibres and myofibrils. The length of the A and of the I substance of the *living* fibre at different degrees of stretch is given in Fig. 2. The values include earlier findings with ordinary light microscopy (1, 2) practically identical with findings by polarized light (standard deviation = 0.06 μ).

At different degrees of stretch, the length of the A and I bands in *fixed* fibres measured in polarized light is practically the same as when measured by electron microscopy. This is seen from Fig. 2, which shows the length of the A and I bands as a function of the length of the sarcomere (A + I).

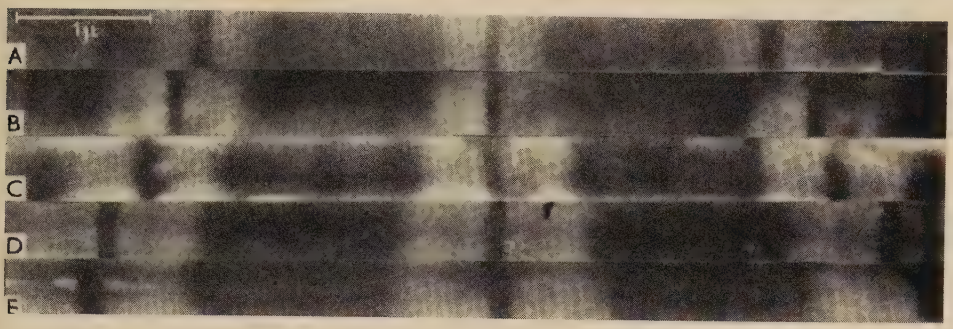

Fig. 3. Electron micrographs of cross striated myofibrils. A: equilibrium length; B–E: at 10, 25, 40, and 60 per cent stretch.

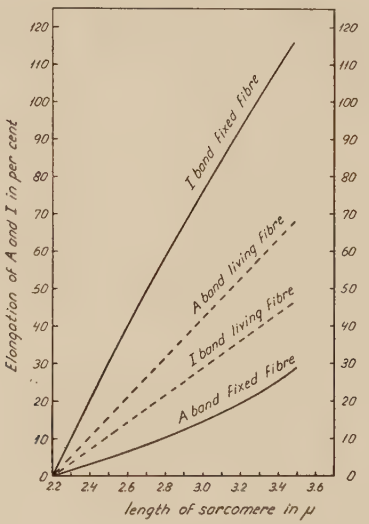

Fig. 4. Elongation of the A and I bands in per cent of their equilibrium length (ordinate) as a function of stretch. Abscissa: length of the sarcomere in μ.

The spread of results from different muscles is small and is approximately the same in the measurements with polarized light and with electron microscopy. Electron micrographs of myofibrils at different degrees of stretch are given in Fig. 3.

The elongations of the anisotropic and of the isotropic bands of the living and fixed muscle fibre at different degrees of stretch in per cent of their equilibrium length are compared in Fig. 4. In the living fibre the length of the A and I bands increases linearly with stretch, the increase in the A band being twelve per cent and in the I band eight per cent for every ten per cent stretch.

In the fixed fibre and myofibril, the elongation of the A and I bands

differs essentially from that of the living fibre. At low degrees of stretch, the A band is elongated much less than the I band, the elongation being 3.5 per cent for A and 22 per cent for I for every ten per cent of stretch of the sarcomere. Above a stretch of about 30 per cent, the elongation of A is 6.5 per cent and of I is 18 per cent for every ten per cent of stretch.

DISCUSSION

The main result of this study was the finding of identical values for the length of the A and I bands of fixed muscle fibres whether using polarized light or electron microscopy. This is the case in spite of the ratio in thickness of a magnitude of 100 : 1. Moreover, measurements of the striations in polarized light and ordinary light were the same. Since inaccuracies in measurement due to the complex nature of the optical conditions are the same in an isolated fibre, whether fixed or alive, these findings indicate that it is possible to obtain correct values for the ratio between the A and the I bands in the living fibre.

There was, on the other hand, a considerable difference in the elongation of the striations in the living and in the fixed fibre with stretch, the ratio of stretch in per cent of A : stretch in per cent of I being 1 : 0.7 in the living muscle at all degrees of stretch; and 1 : 6 to 1 : 2.8 in the fixed muscle, decreasing with increasing degree of stretch. At low degrees of stretch, only a small part of the initially applied stretch is reflected in the A band of the fixed fibre. The major part of the elongation is due to stretch of the I band. The contribution of the A substance to stretch increases above a stretch of 30 per cent, but is still considerably below that of the living fibre.

The difference between living and fixed material is due to a decrease in the extensibility of the A band and an increase in that of the I band both arising from fixation. Thus from length-tension diagrams constructed from the lengths and tensions measured in the living and in fixed fibres, it was found that for loads in the range 0.1–0.5 P_0 the extensibility of the A band was halved while that of the I band was doubled.

In contrast to the findings of A. F. Huxley and Niedergerke (4) and H. Huxley and Hanson (6), the present measurements confirm the relatively high degree of extensibility of the A band in the resting living fibre previously found by Buchthal *et al.* (2) and Buchthal (1). Moreover, measurements in ordinary light microscopy and electron microscopy showed that stretch produced a material elongation of the anisotropic bands in the fixed fibre.

SUMMARY

A method was devised to fix frog muscle at rest, changes in tension induced by shrinkage not exceeding one to six per cent of the maximum tension developed in a tetanus.

Measurements of the length of the A and I bands at different degrees of stretch of the fixed muscle fibre in polarized light and of the myofibril in electron microscopy gave identical results.

Since inaccuracies due to optical conditions are the same in isolated fixed and in living fibres, these findings indicate that it is possible to measure the length of the A and I substance by ordinary microscopy in the living fibre.

Values for the length of the A and I bands in the living fibre at rest were identical as measured by ordinary and by polarized light microscopy and showed a higher extensibility of the A band as compared with the I band.

Fixation introduces a considerable change in the extensibility of the A and I substances. The ratio of stretch in per cent of A : stretch in per cent of I in the living fibre is 1 : 0.70 at all degrees of stretch. With fixation, the ratio is 1 : 6 at low and 1 : 2.8 at higher degrees of stretch.

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Experimental Cell Research, **8**, *336–344 (1955)*

SULPHATE METABOLISM IN THE CARTILAGE OF THE TRACHEA, PINNA AND XIPHOID PROCESS OF THE ADULT MOUSE AS INDICATED BY AUTORADIOGRAPHS

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Received July 2, 1954

Previous investigations by various authors (1–13) have shown that after intraperitoneal application, $^{35}SO_4$ is incorporated in the cartilage and in particular in its chondroitin sulphate. This holds for the adult as well as the developing and the foetal animal (8, 11, 12) and for growing as well as mature cartilage. The concentration of ^{35}S rises steeply during the first hours after injection and subsequently falls slowly. Boström (1) found a maximum uptake of $^{35}SO_4$ in chondroitin sulphate after 24 hours and a fall to half the maximum value after 16 days. Autoradiographic studies (6, 7, 8) have confirmed the uptake of labelled sulphur in cartilage, but the resolving power of the autoradiographs has not been sufficient for the localisation of the labelled sulphur within the cartilage to be studied in detail and no information has been presented as to whether the $^{35}SO_4$ is taken up by the cartilage cells or by the matrix, or whether the uptake varies with different types of cartilage.

The present paper is concerned with an autoradiographic study of labelled sulphur uptake by three different types of adult (i.e. as opposed to the epiphyseal 'growth') cartilage, *viz.* that of the trachea, the pinna and the processus xiphoides of the sternum of young adult mice.

MATERIAL AND METHODS

Single intraperitoneal injections of 250 μ C of $Na_2{}^{35}SO_4$ in 0.5 ml of distilled water were given to 12 male mice (C57 black × C3H—F_1 hybrids) about 3 months old. One or two animals were killed at the following intervals after the injection: 2, 6, 11, 16 and 24 hours, and 4, 6, 7, 10, 11 and 17 days. The pinna, trachea and processus xiphoides were removed and fixed for 1 hour in a mixture of absolute alcohol and

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acetic acid (3:1) and subsequently for 24 hours in formol-saline. After the usual paraffin embedding, sections were cut at 5 μ and prepared for either autoradiographs or various staining methods. Autoradiographs (arg) were made by the stripping film technique with an exposure of about 65 days. After photographic development and fixation, the autoradiographs were allowed to dry for examination by phase-contrast microscopy or were stained with carmalum and mounted in canada balsam after drying at room temperature. Other sections were stained with haematoxylin-eosin, haematoxylin-van Gieson, Weigert's elastin stain, by the periodic acid Schiff technique with and without previous diastase digestion, by Laidlaw's silver method or a modified azan-stain; the same tissues were also taken from control animals and similarly treated. In addition material was also fixed in formol and frozen sections cut and stained with Sudan III. In some further experiments pieces of trachea about 1 mm long were placed in Ringer's solution containing 200 μ C/ml of $Na_2{}^{35}SO_4$ kept at 37° C and fixed at different intervals for autoradiographic studies. Hydrolysis of the slides in normal HCl at 60° C for 10 minutes did not affect the resulting autoradiograph, indicating that the radioactive sulphur responsible for the autoradiograph is organically bound.

RESULTS

The findings common to all three types of cartilage are first described, and the differences between them are recorded afterwards. Figs. 1 to 3 show autoradiographs of sections of tracheal cartilage and illustrate the following behaviour which is common to all three cartilages:

Two hours after the injection (Fig. 1) cartilage cells give a much more intense autoradiograph than the intercellular matrix, the subperichondrial cells or the perichondrium. The arrangement of the cartilage cells into 'chondrones' is clearly seen and within the chondrones the central cells give a less intense autoradiograph than those near the periphery. There is also some variation in intensity of arg in different chondrones.

After sixteen to twenty-four hours the arg over the cartilage cells reaches its maximum and it is also more intense over the matrix. In the central regions of the cartilage the arg over the cells and matrix is less intense than at the periphery but is much heavier than over the subperichondrial cells and the perichondrium. The intensity of the arg over the cartilage cells slowly decreases subsequently, while that over the intercellular matrix increases. The differences between chondrones persist, however, though the arg over the cartilage tends to become more uniform. Fig. 2 shows the appearances after six days. It is obvious that the arg over the cells has weakened and that over the matrix has increased. The same tendency is illustrated in Fig. 3 taken after eleven days. It shows that some of the cells still have a more intense arg than the matrix, but that the difference is sometimes reversed. This is due to the release of ^{35}S from the cells into the matrix. The

differences between chondrones in intensity of the arg persist. The arg over the subperichondrial cells and the perichondrium has also diminished and further decreases are noticed after seventeen days, though positive args are still obtained.

The differences between the three types of cartilage in uptake of radioactive sulphur and in other respects are illustrated in Figs. 4–13 taken 24 hours after injection. The *ear cartilage* (Fig. 4) is of the adipose cellular type in which the cartilage cells are separated by thin trabeculae of matrix and the central part of the cell is occupied by a huge fat droplet (Fig. 5); the cytoplasm and nucleus are forced into a narrow ring lining the capsule. The arg (Fig. 6) appears predominantly over this ring and decreases over the trabeculae of the matrix. This appearance is characteristic for the cartilage of the pinna throughout the period of observation, but the relation between cytoplasmic arg and that of the matrix changes as described above for all types of cartilage.

The anterior part of the *processus xiphoides* consists of hyaline cartilage the posterior region near the tip of a fibrocartilage with mainly elastic fibres in the matrix. In the latter region the matrix is formed by thin trabeculae separating cells with large central fat globules and narrow cytoplasmic rims in which lie the nuclei (Figs. 7 and 8). In the anterior hyaline region the cells are separated by wider partitions of matrix; they contain only small fat globules but more glycogen than the posterior cells. In intermediate zones the two types of cartilage are mingled. The autoradiograph (Fig. 9) is most intense over the cytoplasm, and consequently in the fibrocartilage it appears as a ring (Fig. 10) over the periphery of the cells and in the hyaline cartilage where the cytoplasm is more evenly distributed it covers the whole cell.

The changes in the arg of the *tracheal cartilage* have been described above. For comparison Figs. 11 to 13 are given showing that the cells are separated by greater amounts of matrix, contain only small fat globules and more glycogen; the arg at twenty-four hours is most intense over the cell and less so over the matrix.

The args of Figs. 6, 9 and 13 are taken from the same animal and the

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Fig. 2 (opposite, left). Tracheal cartilage six days after intraperitoneal injection of labelled sulphate. Note the increase in intensity of the arg over the matrix and the decrease over the cartilage cells. × 400.

Fig. 3 (opposite, right). Tracheal cartilage eleven days after intraperitoneal injection of labelled sulphate. Cartilage cells arranged in chondrones are sometimes still more strongly positive than the matrix, but the difference in the intensity of the arg has further decreased and is occassionally reversed. Note the differences in arg between chondrones. × 400.

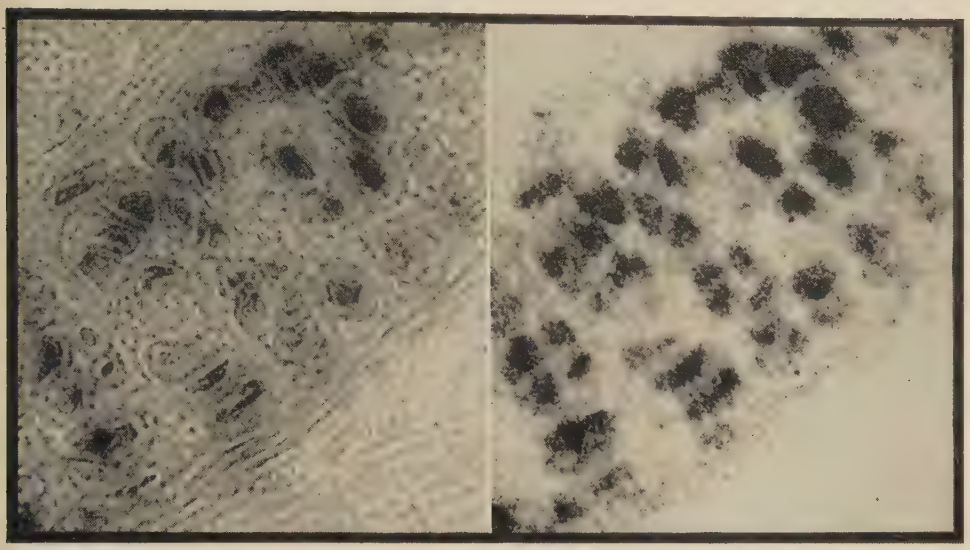
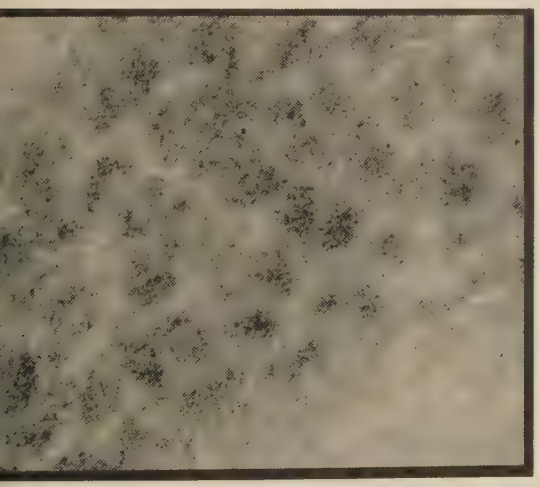


Fig. 1 a. Fig. 1 b.

Fig. 1. Tracheal cartilage two hours after intraperitoneal injection of labelled sulphate (*a*) in phase contrast microscopy, and (*b*) the same section unstained showing the autoradiograph. Note the uptake of ^{35}S by the cartilage cells and much less by the matrix, the arrangement of cells in chondrones with less intense central args, and the differences in intensity of the args of different chondrones. × 400.



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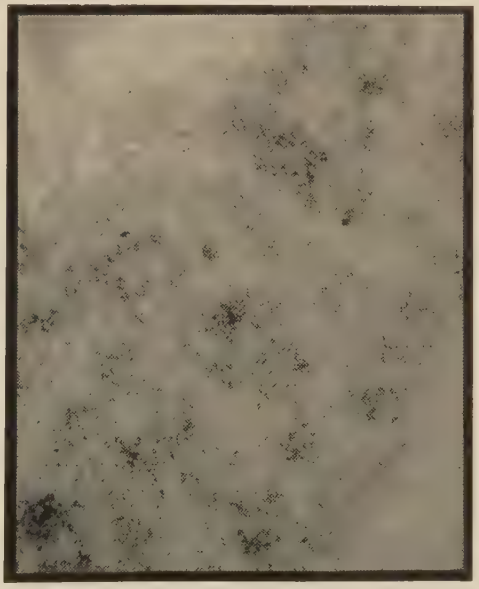
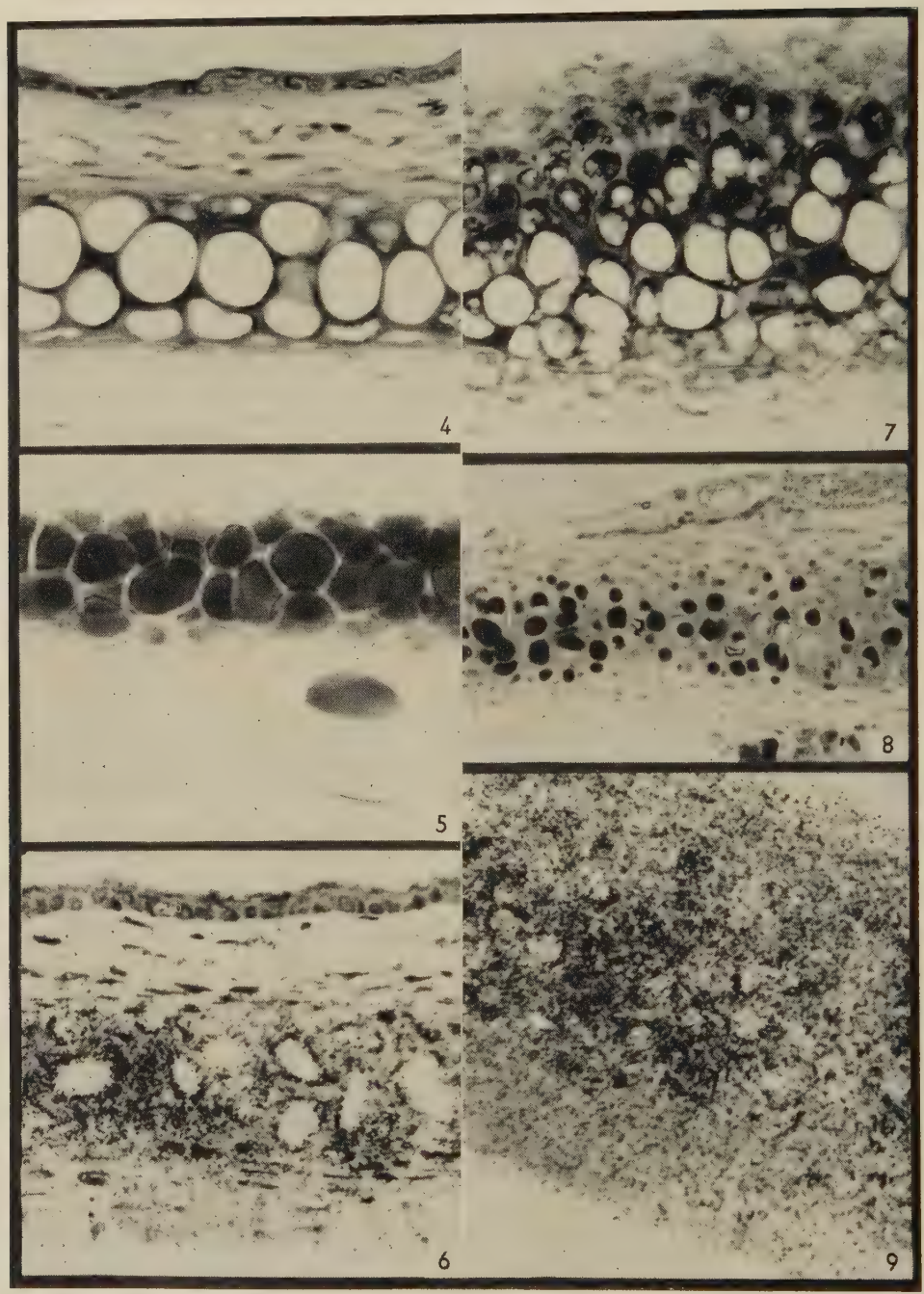


Fig. 3.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sections were mounted on the same slide and treated exactly alike. The illustrations show clearly a difference in the intensity of the arg which increases in the order: pinna → fibrocartilage of xiphoid → hyaline cartilage of xiphoid → tracheal cartilage. It is noteworthy that the amount of matrix and of glycogen increases and that of fat diminishes in the same order. These relative changes in the args of the three types of cartilage remain constant throughout the period of observation.

DISCUSSION

The absence of blood vessels in the cartilage suggests a sluggish metabolism and little activity on the part of the cells once the matrix is formed. This, of course, would not apply to the epiphyseal growth cartilage which reacts to nutritional as well as hormonal factors (Vitamins A and D, and the growth hormone for example). In the relatively static cartilage of the pinna, trachea and xiphoid, on the other hand, little metabolic activity might be anticipated. The fact, however, that after two hours radioactive sulphur appears in the cartilage cells in greater concentration than in any other cells of the body, with the possible exception of the goblet cells of the large intestine and to a lesser extent the mast cells of the derma, suggests a fairly high metabolic activity. Experiments in which pieces of trachea were immersed in Ringer's solution containing $^{35}SO_4$ also showed a rapid uptake of sulphate ions by the cartilage since args made two hours after immersion were essentially similar to those illustrated in Fig. 1 though always weaker in intensity. That the uptake of labelled sulphate is an active process of the cartilage cells is supported by the fact that it is suppressed by CN-poisoning or heat (12).

Fig. 4. Cartilage from the pinna of a mouse injected 24 hours previously with labelled sulphate. Periodic acid Schiff staining. Note the thin trabeculae of matrix and the peripheral ring of cytoplasm and nucleus of the cartilage cells. × 400.

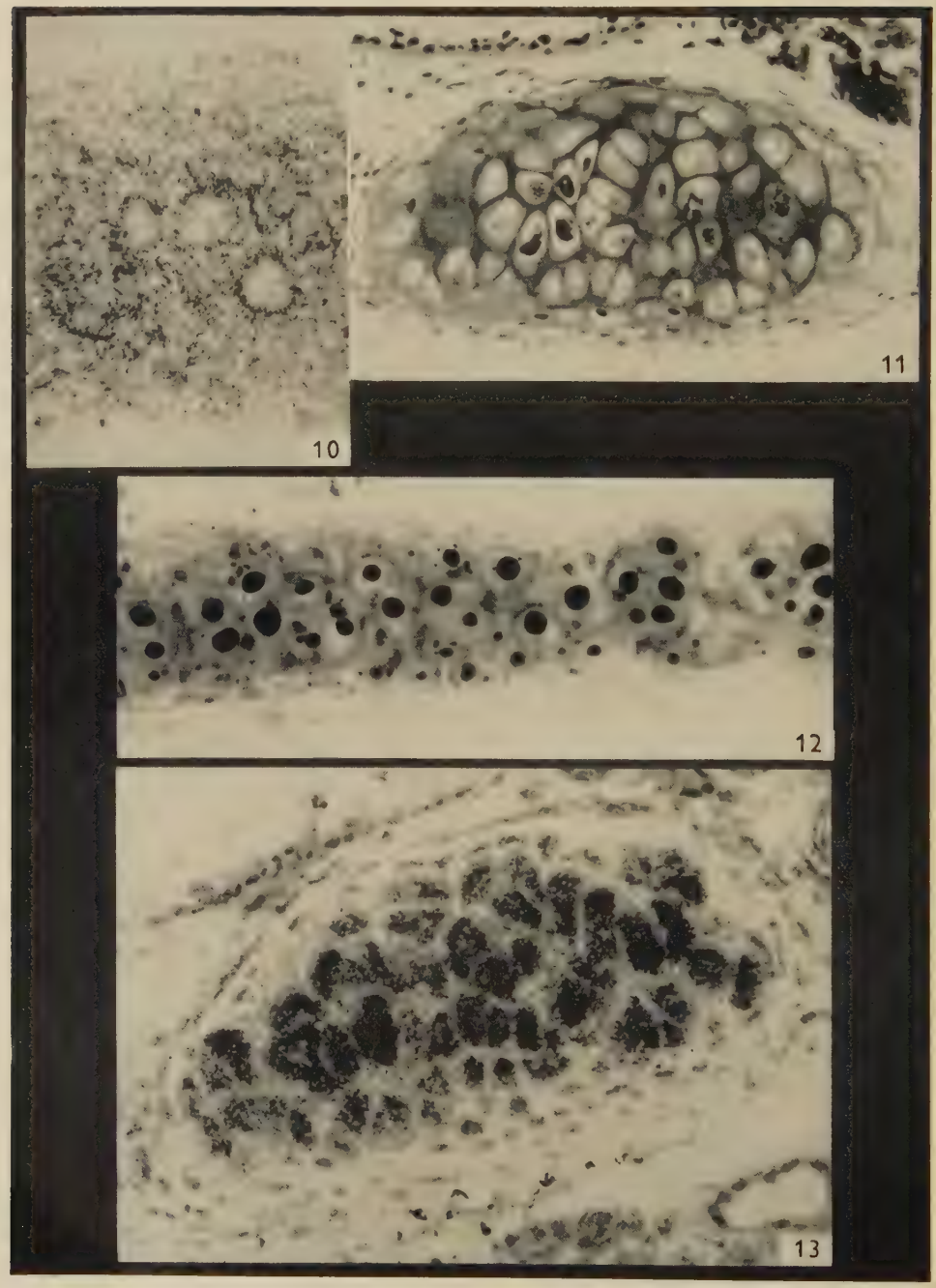
Fig. 5. Cartilage from the pinna of a control mouse. Frozen section after formol-fixation stained with Sudan III. Large fat droplets almost fill the cartilage cells. × 400.

Fig. 6. Cartilage from the pinna of the same animal as in Fig. 4. The arg was prepared after staining the section with haematoxylin-eosin. Note the 'rings' of the arg over the cytoplasmic ring of the cartilage cells and the less intense arg over the thin trabeculae of the matrix. × 400.

Fig. 7. Xiphoid cartilage from the same animal as the pinna in Fig. 4. Periodic acid Schiff staining. Note the presence of adipose as well as of hyaline cartilage cells. × 400.

Fig. 8. Xiphoid cartilage from a control mouse. Frozen section after formol-fixation and staining with Sudan III. Note the difference in fat content of the cells and the thickness of the trabeculae between the fibrocartilage (left) and the hyaline cartilage (right). × 200.

Fig. 9. Xiphoid cartilage from the same animal as the pinna in Fig. 4. The section was stained with haematoxylin-eosin before the arg was prepared. Note the appearance of 'rings' in the fibrocartilage and of 'blobs' in the hyaline cartilage and the difference in intensity of the arg. × 400.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Layton *et al.* (12) have shown that in the intact rat, sulphate is not reduced to sulphur but remains as sulphate though in what form is not known in detail. Our observations demonstrate that at first the cartilage cells rather than the matrix take up the labelled sulphate, store it and then use it for the formation of new chondroitin even in mature 'static' cartilage. This concept is supported by the facts that the intensity of the autoradiograph over the cytoplasm varies with the amount of cytoplasm in the different types of cartilage and that the quantity of cytoplasm parallels that of the intercellular material. Whether the process of continuous new formation extends to the fibrillar basis as well as to the chondroitin of the matrix, cannot be determined by means of autoradiography because of its still limited resolving power.

The storage of sulphate by the cartilage cells and its subsequent release into the matrix explains Boström's observation (1) that the concentration of ^{35}S in the cartilage rises steeply during the first 24 hours after the injection and very slowly falls during the next 16 days.

The variations in intensity of the autoradiographs with chondrones of the tracheal and of the xiphoid cartilages suggest that a chondron may act as a physiological as well as a mechanical unit. The 'chondronic' arrangement of fibrils may thus be only one aspect of the integration of cartilage cells into larger functional units. The greater intensity of the autoradiograph over the peripheral than over the central cells of chondrones after the intraperitoneal injection of labelled sulphate, suggests that the sulphate spreads in the cartilage by diffusion; the rapid diffusion of vital dyes in cartilage is well known.

SUMMARY

Autoradiographic studies were made on the cartilage of the trachea, pinna and xiphoid process of young adult mice at intervals varying from 2 hours to 17 days after the intraperitoneal injection of $Na_2{}^{35}SO_4$.

The labelled sulphate is taken up rapidly by the cartilage cells, stored

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Fig. 10. Xiphoid cartilage of a mouse 2 hours after intraperitoneal injection of labelled sulphate showing the appearance of 'rings' over cells in the fibrocartilage. The slide was stained with carmalum after the arg was prepared. × 400.

Fig. 11. Tracheal cartilage of the mouse of Fig. 4. Periodic acid Schiff staining. The cytoplasm contains glycogen granules. × 330.

Fig. 12. Tracheal cartilage from a control mouse. Frozen section after formol-fixation stained with Sudan III. Note the relatively small amount of intracellular fat. × 385.

Fig. 13. Tracheal cartilage of the same mouse as the pinna in Fig. 4. The section was stained with haematoxylin-eosin before preparing the arg. Note the 'blob'-like appearance of the arg over the cell and the decrease in intensity of the arg over the matrix and towards the perichondrium. × 400.

and slowly released into the matrix. Chondrones differ in their uptake of ^{35}S, and within the chondrones the arg is weaker over the central than over the peripheral cells. The perichondrium and the subperichondrial cartilage cells take up less ^{35}S than the more mature cartilage cells.

The amount of intercellular material increases in the order: pinna → fibrocartilage of xiphoid → hyaline part of xiphoid → hyaline cartilage of the trachea. In the same order the amount of cytoplasm and of glycogen in the cartilage cells increases while that of fat diminishes. The intensity of the arg and its localisation vary with the amount and distribution of the cytoplasm in the different types of cartilage; this accounts for the 'ring'-like appearance in the ear and in the fibrocartilage of the xiphoid and for the more uniform appearance over the cells of hyaline cartilage.

We wish to record our gratitude to Dr. Alma Howard for her generous help with the experiments, to Dr. H. B. Fell, F.R.S. and Dr. G. Popjak for their interest in the work and the criticism of the manuscript, and Mr. G. C. Lenney for some of the photomicrographs.

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Experimental Cell Research, 8, *345–368 (1955)*

CHROMOSOME BREAKAGE IN *ALLIUM* BY 8-ETHOXY-CAFFEINE AND X-RAYS

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Received July 12, 1954

It has been found that a cytological effect strikingly similar to that produced by X-rays may be obtained by treatment with certain chemicals, of which the best known and most thoroughly investigated are the nitrogen and sulphur mustards. Thus these chemicals imitate the effect of X-rays on root tip cells by inducing physiological effects, *e.g.* stickiness of the chromosome matrix, in cells already in division at the time of treatment, and structural chromosome changes in resting cells. Because of their X-ray imitating effects, the chemicals in question are often said to be *radiomimetic*.

Recent detailed analyses, however, have revealed that there also exist important qualitative differences between the effects of X-rays and radiomimetic chemicals. For instance, as first demonstrated by Ford (6), the breaks produced by methyl-bis-(2-chloroethyl)-amine (nitrogen mustard) in root tip chromosomes of *Vicia faba* are localized mainly close to or within the heterochromatic knobs of the st-chromosomes, whereas breaks produced by X-rays are approximately randomly distributed between and within the chromosomes.

This finding was confirmed by Revell in an extensive, comparative study of chromosome breakage by X-rays and radiomimetic substances in *Vicia* (37 and personal commun.). The chemical treatments in this investigation were mainly done with the diepoxide di-(2:3-epoproxypyl)-ether. As regards its cytological effects this chemical behaves very similarly to nitrogen mustard, but it has the advantage of being more stable in aqueous solutions.

In addition to the difference in the localization of breaks, Revell also found other, perhaps more important differences between the cytological effects of X-rays and radiomimetic chemicals. Thus, by means of a double treatment technique, he was able to show that the cell sensitivity to chromosome breakage by X-rays is low at the beginning and high at the end of the resting stage, whereas the sensitivity to chromosome breakage by the diepoxypropylether is at a maximum at the very beginning of resting stage and nil

at the end. Another difference was found in the relative proportions in which the different types of aberrations were obtained various times after the treatments. Thus, if the aberrations were scored as chromatid and chromosome breaks, the chromosome breaks predominated all the time after the chemical treatments, the relative proportions between them and the chromatid breaks remaining about the same. In marked contrast to this, the chromatid breaks in the early fixations after X-ray irradiation were much more frequent than the chromosome breaks. But, as pointed out by Gray (14), "this result may, of course, be conditioned by the inability of the chemical agent to imitate breaks during late interphase".

Revell has never asserted that his results should be valid for radiomimetic substances other than methyl-bis-(2-chloroethyl)-amine and di-(2:3-epoxypropyl)-ether. These are both very reactive chemicals and probably produce their cytological effects by the same mechanism. Quite another type of radiomimetic chemicals is represented by compounds such as maleic hydrazide and the purine derivatives, which have a low chemical reactivity. It is not to be expected that they should act by the same mechanism as do mustards and diepoxides, and therefore the sensitive stage in the cell cycle to these relatively unreactive chemicals might be quite different. In the case of maleic hydrazide, however, McLeish has shown that the sensitive period probably correspond with that for mustards and diepoxides (34). It would thus seem to be a general rule that the interphase chromosomes are most sensitive to breakage by chemicals at the beginning of the resting stage. That this is not necessarily so will appear from the present investigation.

MATERIAL AND METHOD

The radiomimetic chemical used throughout in the present investigation was the purine derivative 8-ethoxycaffeine (EOC), which, as appears from the name, is derived from caffeine by substituting the hydrogen atom in 8-position with an ethyloxy-group. EOC has previously been found to be a potent inducer of structural chromosome changes in root tip chromosomes of *Allium cepa*, *Vicia faba* and *Pisum sativum* (23, 24, 25, 27).

Experimental material consisted of bulbs of common onion *Allium cepa* ($2n=16$). The treatment technique was essentially the same as described previously (24, 25). The roots were grown in about 40 cc of tap water in tubes until they had reached a length of about 2–3 cm. The treatments were performed in such a way that the water was replaced by 40 cc of a 10^{-2} M aqueous solution of EOC. The treatment periods varied between 45 and 60 minutes, the standard treatment being 50 minutes. At the end of the treatment the roots were replaced in water. Fixations were done at short intervals during the first day after the treatment. All experiments were

performed in the dark. If not otherwise stated, the temperature during treatment was 20° C.

The X-ray treatments were given at 165 kV, filter 1 mm Al, intensity 20.1 r/min. The roots were irradiated wet in the air and then replaced in water.

The root tips were fixed in alcohol-acetic acid, 3:1, and prepared as Feulgen squashes. The slides were made permanent by removing the cover slips after the slides had been frozen on dry ice, the slides then being passed through alcohol and xylol and mounted in Canada balsam.

When scoring the changes no attempt to distinguish between chromosome and isochromatid breaks have been made. When both chromatids are broken at the same point, the aberration is scored as an isolocus break according to the terminology of Thoday (41).

INTERPHASE SENSITIVITY TO CHROMOSOME BREAKAGE BY EOC AND X-RAYS

After a number of preliminary experiments, the EOC concentration and period of treatment to be used in the main experiments were fixed to 10^{-2} M and 50 minutes, respectively. This treatment produced a considerable number of chromosome changes but had only slight inhibitory effect on cell division.

This EOC dose was now given to 8 bulbs. Fixations were made from 0 to 24 hours after the treatment, in the beginning with short intervals. The result of the experiment appears from Table I and Fig. 1, each figure being based on 100 analysed anaphases.

Probably as a result of heavy stickiness, the cell divisions were stopped in metaphase configuration for about 20 minutes after the treatment. Thirty minutes after the treatment separation of sister chromatids at anaphase was possible, the stickiness effect being still very pronounced, however. In a few cells changes of the pseudo-chiasma type were seen, but because of the heavy stickiness it was not possible to decide if they were true pseudo-chiasmata or had arisen as a result of stickiness.

One hour after the treatment most of the cell divisions analysed were quite normal. A few anaphases with sticky-bridges (9 per cent), pseudo-chiasmata (1 per cent), and fragments (3 per cent) were observed.

Two hours after the treatment there was again an increase in aberrant cells. Among the changes which could be observed in low frequencies were misdivisions of the centromeres. But the predominant changes were now those of the pseudo-chiasma type. There has been considerable disagreement about the nature of these changes, which have been called pseudo-chiasmata by Levan and Tjio (32) because of their resemblance to the meiotic chiasmata. The correct explanation to their origin has probably been given by

TABLE I

Aberrations observed at different times after treatment with EOC (10^{-2} M/50 min.).

Duration of recovery period (hours)	Per cent of abnormal anaphases			Number of fragments per anaphase	Number of bridges per anaphase
	Sticky anaphases	Anaphases showing subchromatid breakage	Anaphases showing chromatid a. isolocus breakage		
1/2	66	5.5	1.1	0.01	0.01
1	9	1	3	0.06	0.01
2	4	46	10	0.11	0.04
3	—	13	42	0.76	0.10
4	—	5	50	1.25	0.17
6	—	1	22	0.69	0.10
8	—	—	12	0.18	0.10
12	1	—	5	0.06	0.04
18	—	1	16	0.28	0.14
24	—	—	15	0.16	0.06

LaCour and Rutishauser (29). According to these authors, the pseudo-chiasmata are due to subchromatid breakage and reunion. The data of LaCour and Rutishauser further indicate that "regularly subchromatid breaks are induced by treatment during prophase but not during the resting stage and metaphase". This conclusion is in fact supported by the results obtained in the present investigation. By means of the binucleate cell technique it was found that prophase and metaphase together last for about 2 hours in root tip cells of *Allium cepa*. In EOC-treated roots the cell divisions are completely arrested at metaphase for about 20 minutes after the treatment, the anaphase separation probably being somewhat delayed for an additional 30 minutes. This means that it takes about 2 1/2 to 3 hours before a cell, which during the treatment was in late interphase or early prophase, has reached the anaphase. From this it follows that the cells scored 1 hour after the treatment probably had been treated at late prophase. Only 1 per cent of these showed subchromatid breakage. One hour later, i.e. 2 hours after the end of the treatment, the anaphases represent such cells as were treated in early or middle prophase and now a considerable number of pseudo-chiasmata were observed. But 3 hours after the treatment, when such cells as were in late interphase, or possibly very early prophase, during the treatment had reached anaphase, only a few pseudo-chiasmata were found. Thus it seems

clear that pseudo-chiasmata or subchromatid breaks are in general produced by treatment during prophase.

When the subchromatid breaks were at their maximum 2 hours after the treatment, only a few chromatid and isolocus breaks were observed. However, 3 hours after the end of the treatment both chromatid and isolocus breaks were frequent. Four hours after the treatment these aberrations occurred with maximum frequencey. This maximum was followed by a rather rapid reduction in the number of aberrations so that 12 hours after the treatment only a few abnormal cells were found. But the root tips from the two last fixations (18 and 24 hours after the treatment) contained again a somewhat greater number of abnormal cells.

With the results of the investigations of Revell on the interphase sensitivity to X-rays and diepoxypropylether in mind, the observed frequencies of structural chromosome changes various times after EOC-treatment are rather interesting. They seem to indicate, that the root tip cells of *Allium* are most sensitive to chromosome breakage by EOC at late interphase. This again should mean that the interphase sensitivity to EOC is very similar to that to X-rays, but quite different from the interphase sensitivity to the other radiomimetic chemicals hitherto studied.

Since Revell's investigations were performed with *Vicia* and those of the present author with *Allium*, a comparison between the results obtained is not entirely satisfactory. Therefore some X-ray experiments with *Allium* were also performed.

Two series of ten bulbs in each were irradiated with 75 and 100 r respectively. The results of the irradiations appear from Tables II and III.

The effects on dividing cells produced by these treatments were considerably weaker than those produced by EOC. After irradiation with 75 r, only a very slight stickiness effect and very few pseudo-chiasmata were obtained. 100 r produced a somewhat stronger effect, but nothing comparable with the corresponding changes produced by EOC. The stickiness effect seems to appear later and to last longer in the X-ray experiments.

Both in the 75 and 100 r series, structural chromosome changes are first obtained 3 hours after the treatment. As after EOC-treatments, they occur with maximal frequency 4 hours after the treatment. The structural chromosome changes do not, however, disappear as rapidly in the X-ray as in the EOC experiments and there is nothing corresponding to the minimum observed 12 hours after EOC-treatments. If only the effect 4 hours after the treatment is considered, 75 r produces about the same amount of chromosome damage as does a 50 minutes treatment with 10^{-2} M of EOC.

TABLE II

Aberrations observed at different times after treatment with 75 r of X-rays.

Duration of recovery period (hours)	Per cent of abnormal anaphases			Number of fragments per anaphase	Number of bridges per anaphase
	Sticky anaphases	Anaphases showing subchromatid breakage	Anaphases showing chromatid a. isolocus breakage		
1/2	—	1	—	—	—
1	1	—	—	—	—
2	3	10	—	—	—
3	—	—	44	0.88	0.06
4	—	—	68	1.23	0.09
7	—	—	30	0.47	0.07
10 1/2	—	—	30	0.48	0.11
24	—	—	30	0.35	0.12

TABLE III

Aberrations observed at different times after treatment with 100 r of X-rays.

Duration of recovery period (hours)	Per cent of abnormal anaphases			Number of fragments per anaphase	Number of bridges per anaphase
	Sticky anaphases	Anaphases showing subchromatid breakage	Anaphases showing chromatid a. isolocus breakage		
1/4	—	—	—	—	—
1	7	—	—	—	—
2	26	6	—	—	—
3	4	15	36	1.10	0.02
4	1	7	72	2.32	0.10
7	—	—	60	1.17	0.16
10 1/2	—	—	51	0.84	0.14
24	—	—	35	0.50	0.13

The results are shown graphically in Fig. 2, the effect here being scored as fragments (= acentrics = C_0) and bridges (= dicentrics = C_2) per cell. For comparison the effect of 10^{-2} M of EOC (50 minutes treatment) is included.

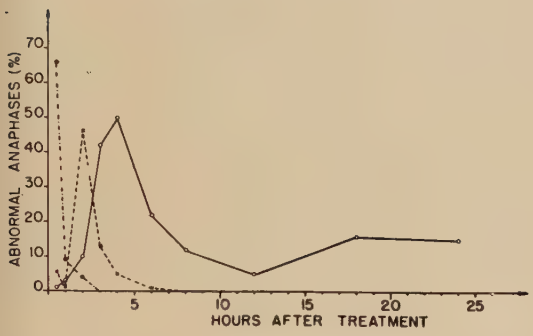
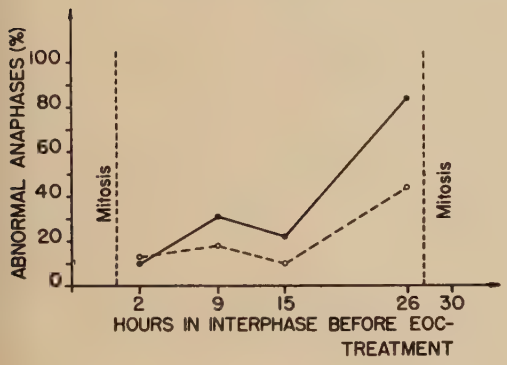
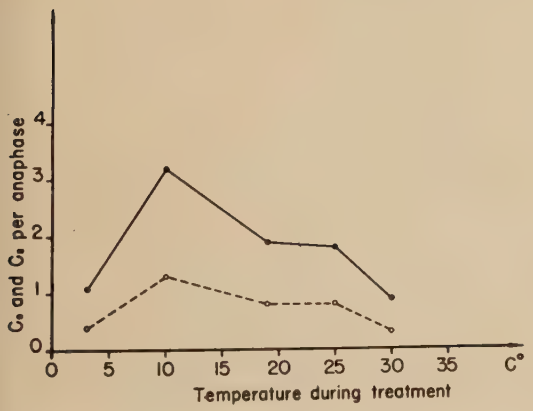


Fig. 1. The effect of 8-ethoxycaffeine (EOC) after recovery periods of varying duration.

•—•—• Stickiness
×--×--× Subchromatid breaks
○—○—○ Chromatid and isolocus breaks





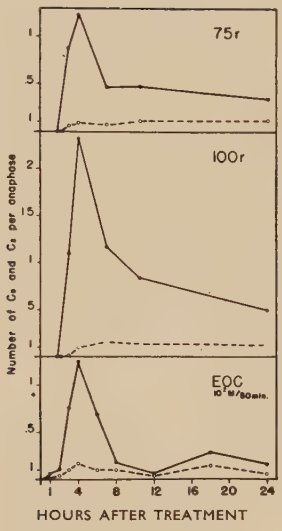


Fig. 2. The frequencies of fragments (C_0) and bridges (C_2) after treatment with X-rays and EOC.

•—• C_0
○--○- C_2

Fig. 3. Interphase sensitivity to EOC.

•—•—• Binucleate cells
○--○--○ Mononucleate cells

Fig. 4. The effect of EOC at different temperatures.

•—•—• Fragments (C_0) per anaphase
○--○--○ Bridges (C_2) per anaphase

It appears from the figure that the similarities are not restricted to the shape of the curves; also the proportions between C_0 and C_2 various times after the treatments seem to be the same in the EOC and X-ray experiments. Since interchanges are rather rare in *Allium* after low dose treatments, most bridges have arisen as a result of sister chromatid union in broken chromosomes and they should therefore give a rough idea of the frequency of isolocus breaks. If this assumption is correct, the frequency of isolocus breaks should be comparatively unaltered during the experiment, whereas the chromatid breaks, most of which give fragments but no bridges at anaphase, should be very frequent at the beginning of the experiment but rather few at the end of it.

Scoring of breaks is usually done at metaphase. This is, however, very difficult in *Allium*, since the chromosomes are numerous and close together in the metaphase plate. The difficulties can be avoided by treating the cells to be scored with colchicine before fixation. Since colchicine inactivates the spindle apparatus and induces a delay of the division of the centromere, the root tips after a colchicine treatment contain a great number of cells with their metaphase chromosomes freely distributed in the cytoplasm. A disadvantage of the method is that the metaphases obtained in this way represent a mixture of cells treated at different interphase stages. In order to make the colchicine treatment effective and to obtain a sufficient number of cells for scoring, it is necessary to treat the root tips for several hours.

In connection with the EOC and X-ray experiments (75 r) described above, some onions were treated before fixation with a 0.05 per cent aqueous solution of colchicine for 3 hours. The first fixations were made 5 $^1/_2$ hours after the EOC and X-ray treatments, respectively. The root tips in these fixations therefore contained metaphases, which had been treated 2 $^1/_2$ to 5 $^1/_2$ hours before reaching this stage. The second time fixations were made was 9 $^1/_2$ hours after the treatments and the last fixations were made 22 and 27 hours after the EOC and the X-ray treatments, respectively.

The results of these experiments appear in Tables IV and V. The frequencies of chromatid breaks are in both cases very high in metaphases fixed 5 $^1/_2$ hours after the treatments and low in metaphases from the last fixation. As anticipated on the basis of the C_0 and C_2 frequencies in Fig. 2, chromatid breakage predominates at the beginning of the experiment and isolocus breakage at the end of it. If only the first and the last fixations are considered, the proportion between chromatid and isolocus breaks is thus about the same in the EOC and the X-ray experiments. In the second fixation, however, the isolocus breaks predominate in the EOC but not in the X-ray

Chromosome aberrations after treatment with EOC (10^{-2} M/50 min.).

Time after treatment (hours)	Number of metaphases analysed	Abnormal metaphases		Aberrations per 100 cells			
		Number	Per cent	Chromatid breaks	Isolocus breaks	Chromatid exchanges	Isolocus exchanges
2 1/2– 5 1/2	115	63	54.8	53.9	22.6	5.2	—
6 1/2– 9 1/2	310	44	14.2	4.2	10.6	0.6	—
19–22	344	81	23.5	5.1	20.4	1.2	0.3

TABLE V

Chromosome aberrations after treatment with 75 *r* of X-rays.

Time after treatment (hours)	Number of metaphases analysed	Abnormal metaphases		Aberrations per 100 cells			
		Number	Per cent	Chromatid breaks	Isolocus breaks	Chromatid exchanges	Isolocus exchanges
2 1/2– 5 1/2	53	43	81.1	118.9	30.2	20.8	—
6 1/2– 9 1/2	86	64	74.4	65.1	38.4	7.0	—
24–27	279	84	30.1	10.0	22.9	1.1	0.4

TABLE VI

The radiomimetic effect of EOC at different stages of interphase.

Time in resting stage before EOC treatment (hours)	Duration of recovery after EOC treatment (hours)	Binucleate cells				Mononucleate cells			
		Number of nuclei analysed	Abnormal anaphases (per cent)	Number of fragments per nucleus	Number of bridges per nucleus	Number of nuclei analysed	Abnormal anaphases (per cent)	Number of fragments per nucleus	Number of bridges per nucleus
2	27.5	42	10.5	0.05	0.05	100	13	0.18	0.03
9	20.5	100	31	0.41	0.04	100	18	0.20	0.08
15	14.5	50	22	0.30	0.12	100	10	0.12	0.07
26	3.5	50	84	1.88	0.30	100	44	0.98	0.17

experiment. The decrease in the frequency of chromatid breaks is thus less rapid after X-ray irradation than after EOC treatments.

The percentage of chromatid breaks involved in interchanges is low in both the EOC and X-ray experiments. This low frequency of interchanges is perhaps correlated with the fact that the chromosomes in *Allium* lack visible heterochromatin. In *Vicia faba*, where heterochromatic chromosome segments are common, the frequency of interchanges is high both after X-ray and chemical treatments. It has been proposed (34, 37) that the heterochromatin favours reunion in a very specific way by keeping the chromosomes together in chromocenters.

The quantitative differences between the figures obtained in the colchicine experiments on the one hand and in the ordinary experiments on the other, are mainly due to the different methods of scoring. Thus in contrast to an ordinary experiment, the effect in a colchicine experiment is scored in a mixture of cells, treated at different times before metaphase. Another reason for the discrepancies found is that, particularly after X-ray irradiation, a significant number of the achromatic points scored as chromatid breaks in the colchicine experiments in fact are not breaks at all: that is, they do not result in fragmentation at anaphase. Finally after treatments with low doses of EOC, there is often a large variation between the effects produced in roots from different bulbs.

From the data presented above it can be concluded that the root tip cells of *Allium cepa* are most sensitive to chromosome breakage by both EOC and X-rays at the end of interphase, and that the frequencies of chromatid and isolocus breaks obtained different times after treatment also are remarkably similar in the two cases. In these respects the effect of the chemical EOC is thus more similar to that of the physical X-ray treatment than to the effect of other chemicals such as mustards, diepoxides and maleic hydrazide.

THE BINUCLEATE CELL TECHNIQUE FOR DETERMINATION OF INTERPHASE SENSITIVITY

In the experiments described above, the estimations of the stages at which the analysed cells had been treated, were based only on the time which elapsed between treatment and fixation. This method is not quite satisfactory, however, because all cells do not divide with equal speed, particularly after a treatment, which may have affected certain cells more than others and therefore also delayed them more.

Revell has developed a technique, which enabled him to make a more

accurate estimation of the time in resting stage at which the cells had been treated with X-rays or diepoxypropylether. This technique "involves the measurement of two times: as well as the usual time between treatment and fixation, an estimate is also made of the time from before the previous mitosis up until treatment. This is obtained by marking all cells at this previous stage with micronuclei by means of a previous treatment; only these cells are later analysed" (37).

In collaboration with Revell efforts were made at the Chester Beatty Institute for Cancer Research to determine interphase sensitivity to chromosome breakage by EOC by means of this technique. The marking was done with a diepoxypropylether treatment. The experiments gave the expected result: cells in late interphase were most sensitive to EOC. The double treatment resulted, however, in a rather pronounced delay of mitosis, which rendered the interpretation more difficult. Because of these difficulties with the technique of Revell, another marking technique was worked out.

In addition to their ability to produce chromosome aberrations, the purine derivatives have another characteristic cytological effect: they inhibit the cell wall formation between daughter nuclei at telophase. This effect has been studied particularly by French workers (12, 33) and has by them been called the stathmodiérèse reaction.

In the new marking method the stathmodiérèse reaction is made use of. This was possible because in *Allium* cell wall formation is inhibited by lower concentrations than those which produce chromosome breakage.

The marking in the present experiments was obtained by treating root tips for 4 hours with a 5×10^{-3} M solution of theophylline. This treatment did not produce any chromosome breaks, nor did it delay the divisions of the cells. But as a result of the treatment a considerable number of binucleate cells were formed. The first binucleate cells were observed about 2 hours after the beginning of the treatment and $^1/_2$ hour subsequent to the treatment cell walls were formed again.

By repeated fixations during the next 30 hours, the division cycle of the binucleate cells could be followed. It was found that in February at 19° C, the interphase lasts between 20 and 26 hours, the period from early prophase to anaphase about 2 hours, and anaphase and telophase together about $^3/_4$ hour. The time of the total mitotic cycle of the binucleate cells varies thus between 23 and 29 hours. The divisions of the two nuclei are absolutely synchronized.

There is some evidence that the duration of the mitotic cycle in the normal mononucleate cells is of the same order. Thus after short EOC treatments of

the type described above, prophases with micronuclei have never been observed earlier than about 30 hours after the treatment. As is well known, micronuclei arise at telophase from such fragments as are not incorporated in either of the two daughter nuclei. Since practically no fragments have been observed at anaphase during the first 2 hours after the EOC treatment, and since the division itself lasts for about 3 hours, the observations indicate that the duration of interphase is about 25 hours and that of the whole mitotic cycle about 28 hours.

After similar EOC-treatments in *Vicia*, the first binucleate cell-divisions (EOC also inhibits cell wall formation, but this reaction is not as easily separable from the effects on the chromosomes as in the case of theophylline) appeared about 20 hours after the treatment. The figure "20 hours" thus found for the duration of interphase in *Vicia*, is in very good agreement with those found by other workers using different methods (17, 19, 37).

Since the binucleate cell technique gives correct information as to the length of the mitotic cycle in *Vicia*, it seems reasonable to assume that it does so also in *Allium*. In any case, the figures obtained by Laughlin (31), according to which the whole mitotic cycle should be completed in about 4 hours at 20° C, can certainly not be correct. Darlington and LaCour (4) have estimated the average length of the resting stage in *Allium* root tips to about 1.5 days at 24° C. This figure fits in better with those obtained by the present author, although it seems to be somewhat too high.

The interphase sensitivity to chromosome breakage by EOC was tested in the following way:

In the root tips of 10 bulbs binucleate cells were produced by means of a theophylline treatment as described above. Two of the bulbs were used as controls, the remaining 8 were treated at different times with a 10^{-2} M solution of EOC for 50 minutes. Of the 8 bulbs, the first 2 were treated 2 hours after the theophylline treatment, when the binucleate cells were in early resting stage, and the last 2 bulbs were treated when the binucleate cells were in late interphase or very early prophase, this occurring 26 hours after the theophylline treatment. EOC treatments were also performed 9 and 15 hours after the theophylline treatment. All roots were fixed at the same time, i.e. $3\ ^1/_2$ hours after the last of the four EOC treatments. Thus, the whole experiment lasted about $34\ ^1/_2$ hours. The result of the experiment appear from Table VI and Fig. 3.

Data showing the effect on the mononucleate cells in the same root tips have been included as a comparison. The effect on the binucleate cells is considerably stronger than that on the mononucleate ones, which indicates that

theophylline treatment during mitosis renders the cells more sensitive to subsequent EOC treatments. The shapes of the two curves in Fig. 3 are very similar, however. This means that the conclusions regarding interphase sensitivity to EOC drawn from the ordinary experiments were on the whole correct. The chromosomes are most sensitive to breakage by EOC at the very end of interphase, and they seem to be relatively unsensitive in the middle of this stage, but more sensitive again in the beginning of interphase.

Prior to the experiment, the low effect obtained in cells fixed $^1/_2$ day after the treatment was believed to be a result of a selection of normal cells, the EOC affected cells at this stage being more delayed than the relatively unaffected ones. This interpretation was supported by the fact that aberrant cells occur with a higher frequency in roots fixed $^3/_4$ to 1 day after the treatment, than in the $^1/_2$ day fixations. If the explanation mentioned should be correct, a higher percentage of aberrant cells should be expected later when the affected and delayed cells have reached mitosis. The mitoses fixed at these times should thus, according to the hypothesis, represent a mixture of cells treated at different periods of interphase.

But when the cells are marked by the binucleate cell technique, it is known at which stage of interphase the cells are treated. The aberrant cells fixed 18 to 28 hours after the EOC treatment must represent the effect on cells in early interphase, and there is therefore no doubt that this stage is really sensitive to EOC, although considerably less so than the late stages of interphase. This is perhaps the most important result of the marking experiment. The low effect at the middle of interphase could, of course, still be a result of a selection of normal cells, although it now seems less likely. At the present time the author is more inclined to believe that in the middle of resting stage either the nuclear envelope is relatively impermeable to EOC or in fact the chromosomes are unbreakable by this agent.

LOCALIZATION OF BREAKS

In *Vicia faba* X-ray induced single breaks show an apparently random distribution, except that there are few in the heterochromatin (37). In other plants too there is reported a rather even distribution of X-ray induced breaks. Gottschalk (13) found, however, the centromere regions of the chromosomes of *Solanum lycopersicum* particularly sensitive to X-ray breakage.

In conncetion with the present investigation no attempts have been made to analyse the localization of the breaks produced by X-rays and EOC in *Allium*. It is, however, quite obvious, that while the breaks and points of

interchanges after X-ray irradiation appear to be rather evenly distributed between and within the chromosomes, those after EOC treatments occur more frequently than could be expected from a random distribution in the attachment threads of the satellites (Fig. 5). A similar localization of EOC-induced breaks has previously been observed in *Vicia* (27) and in *Pisum* (25). In the latter plant, breaks were also very frequent in the centromeric regions.

As an explanation of the localization of breaks observed after EOC treatments, it has been suggested that EOC has a tendency to concentrate on or within the nucleolus (25). There should therefore be a higher frequency of breaks in chromosome segments in contact with the nucleolus, where the EOC concentration is assumed to be high, than in those lying in other parts of the nucleus.

MODIFYING FACTORS

Oxygen tension. Some years ago Thoday and Read (42) observed, that if root tips of *Vicia faba* were irradiated with X-rays in nitrogen atmosphere, the yield of aberrant cells (cells with bridges and fragments) was only about $^1/_3$ of that obtained after irradiation in oxygen. In another paper Thoday and Read (43) reported that not only the frequency of structural chromosome changes, but also the physiological effect produced was considerably weaker in the absence of oxygen. When α-radiation was used in place of X-rays, the influence of anaerobiosis on the production of chromosome aberrations was very much smaller.

A similar effect of oxygen tension in the production of chromosome breakage by X-rays has since then been found by several workers, *e.g.* Giles and Riley (10, 11), Gray *et al.* (16), using different tissues and organisms. The influence of the oxygen tension on the physiological effect of X-rays has recently been pointed out by Swanson and Schwartz (40).

According to Thoday and Read (43), their results suggest "that hydrogen peroxide has some influence on the process involved in chromosome structural change". Hydrogen peroxide is formed in water by X-ray irradiation only if there is dissolved oxygen present. The production of hydrogen peroxide in water by α-rays is, on the other hand, quite independent of the dissolved oxygen.

The influence of oxygen tension on the yield of chromosome aberrations produced by X-rays and α-particles, seems thus to favour the opinion that chemical steps intervene between ionization and chromosome damage. Most workers in the field seem to accept this as an established fact, although

some doubts exist regarding the significance of hydrogen peroxide. Another product of X-ray irradiation in water, the hydroperoxyl radical (HO_2) is now suspected to play the major role in the production of chromosome damage (9).

Since it is rather difficult to imagine how EOC should be able to produce free radicals of the HO_2 type, it would not be expected that the oxygen tension should have any influence on the degree of chromosome damage produced by EOC. Experiments were, however, performed to confirm this.

The methods used and the results obtained have been briefly described in a previous paper (26). The experiments were performed in the usual way, except that nitrogen or oxygen was bubbled through the solutions in which the onion roots were immersed. In order to minimize the back diffusion of air, the bulbs and the upper parts of the tubes containing the solutions were enclosed in bags of plastic, with small opening for the egress of the gas in question. In this way the roots were kept in oxygen or nitrogen atmosphere during the EOC treatment as well as during a short time (15–30 min.) before and after the treatment. The concentration of EOC was the usual one of 10^{-2} M. Identical treatments were always given to 2 bulbs.

In the two experiments performed the treatment conditions were as follows:

Experiment 1: period of treatment: 1 hour; treatment temperature: 18° C; the bulbs were kept 30 min. before and 15 min. after the EOC treatment in oxygen or nitrogen.

Experiment 2: period of treatment: 45 min.; treatment temperature: 20° C; the bulbs were kept 15 min. before and 15 min. after the treatment in oxygen or nitrogen.

In the first experiment some slides were made from root tips fixed 40 min. after the treatment in order to get information of the physiological effect produced. It was found that the stickiness effect produced by EOC was very significantly influenced by the oxygen tension. A normal treatment (in air) produces, as shown by Fig. 1 (page 351) a very strong stickiness, which, however, disappears rather rapidly, so that 1 hour after the treatment less than 10 per cent of the cells contain sticky chromosomes. When, however, the roots were treated in nitrogen, hardly any stickiness at all was produced. On the other hand, when the treatment was performed in oxygen, a stronger stickiness effect than after an ordinary treatment was produced, and, as appeared from later fixations, it also lasted for a longer time. Thus, the physiological effect produced by EOC showed the same dependence on oxygen tension as that produced by X-rays.

Tables VII and VIII show that also the structural chromosome changes

Table VII

The effect of EOC (10^{-2} M/1 h/18°C) in nitrogen and oxygen.

Duration of recovery period (hours)	Nitrogen			Oxygen		
	Abnormal anaphases (per cent)	Number of fragments per anaphase	Number of bridges per anaphase	Abnormal anaphases (per cent)	Number of fragments per anaphase	Number of bridges per anaphase
4	5	0.11	0.02	40.9	1.75	0.19
	3	0.03	0.01	11	0.28	0.04
8	12	0.26	0.06	90.7	2.47	1.20
	13.4	0.18	0.07	67.7	2.34	0.33
24	4	0.06	0.02	43	0.76	0.27
	5	0.07	0.02	30	0.56	0.21

Table VIII

The effect of EOC (10^{-2} M/45 min./20°C) in nitrogen and oxygen.

Duration of recovery period (hours)	Nitrogen			Oxygen		
	Abnormal anaphases (per cent)	Number of fragments per anaphase	Number of bridges per anaphase	Abnormal anaphases (per cent)	Number of fragments per anaphase	Number of bridges per anaphase
2	3	0.03	0.01	15	0.20	0.03
4	18	0.27	0.02	30.3	0.65	0.07
	11	0.13	0.02	61	1.18	0.22
10	5	0.05	0.03	19	0.27	0.11
	1.9	0.02	—	18	0.21	0.08
24	10	0.14	0.04	21	0.27	0.07
	3	0.03	—	16	0.26	0.09

produced by EOC are influenced by the concentration of oxygen during the treatment.

The figures in Tables VII and VIII are based on at least 50, in most cases 100, analysed anaphases. In the first experiment (Table VII) the maximum effect appears later than usual, i.e. 8 hours instead of 4 hours after the treatment. The reason for this is that the temperature is lower and the period of treatment longer than usual, both factors tending to decrease the speed

of mitosis and therefore to delay the appearance of the chromosome aberrations.

The difference between the frequencies of chromosome aberrations produced by EOC in nitrogen on the one hand and in oxygen on the other, is considerable, and although the variation between the two bulbs treated in oxygen is great, there can be no doubt about its significance. At the maximum 8 hours after the EOC treatment, the mean effect of EOC in oxygen is about six times stronger than that in nitrogen. It may be added, that treatments with oxygen or nitrogen alone for a period of 105 min. did not result in any kind of chromosome damage.

The second experiment was performed under more normal conditions as regards temperature and duration of treatment, and therefore the maximum effect is obtained, as usual 4 hours after the end of the treatment. The influence of oxygen on the EOC effect is not as pronounced as in the first experiment, but it is still quite significant. In fact, if the calculations are based on mean values, the effect in nitrogen is about $^1/_3$ of that in oxygen, which is the same difference as that found by Thoday and Read in their X-ray experiments.

In the case of X-rays it is believed "that oxygen makes available certain radicals produced from the decomposition of water which would otherwise be lost" (15). Since a similar explanation in the case of EOC is out of question, oxygen probably influences the effect of EOC on chromosomes by altering the biochemical processes of the cell in such a way that the breakability of the chromosomes increases. But if this is the explanation in the case of EOC, it should perhaps not be unreasonable to assume that in a large measure oxygen exerts its action in a similar way in the case of X-rays. This suggestion seems to be supported by the discovery of Conger and Fairchild (3), that oxygen itself is able to produce chromosome breakage, and also by the finding of King *et al.* (28) that exposures to the cytochrome oxidase poison carbon monoxide before and during X-ray irradiation increases the frequency of chromosome aberrations.

The following explanation would perhaps be more consistent with the radiochemical interpretation of the influence of oxygen in the case of X-rays. EOC cannot decompose water yielding free radicals. But it could perhaps cause an accumulation of hydrogen peroxide in the cell or nucleus by inhibiting such enzymes as are responsible for the neutralization of peroxide normally formed in the cell as a result of oxidative processes. An enzyme of this type is peroxidase, which has haematin as a prosthetic group. In fact an inhibition of this enzyme by EOC could be expected, since J. Keilin

(21) has shown that caffeine derivatives have a solubilizing effect on haematins and that this solubilizing effect is correlated with a complex formation between purine and haematin, the complex being recognized by its different absorption spectrum. While EOC according to this hypothesis should act by preventing the destruction of hydrogen peroxide, the effect of oxygen should be to increase the formation of peroxide (see King *et al. loc. cit.*).

The effect of EOC on horse radish peroxidase has kindly been tested by Dr. K. G. Paul, Biochem. dept., Karolinska Institutet, Stockholm. The following results were obtained:

1. the absorption spectrum of peroxidase was not altered in the presence of EOC, which indicates that no molecular complex was formed.
2. EOC was neither in the presence nor in the absence of peroxidase oxidized by hydrogen peroxide.
3. the mesidine test (36) showed that EOC in a concentration of 3×10^{-3} M does not influence the activity of horse radish peroxidase.

Thus the peroxide hypothesis was not supported by the results of the experiments of Dr. Paul.

More detailed investigations of the effect of oxygen on the yield of chromosome aberrations produced by EOC are planned. Further consideration on the oxygen effect may therefore be postponed until the data from these experiments are available.

Temperature. That the yield of chromosome aberrations produced by X-rays in *Tradescantia* microspores is dependent on temperature during and immediately after the treatment was shown by Sax and Enzmann (39). The flowerbuds were irradiated at temperatures varying from 3 to 38° C and it was found that, as a rule, the lower the temperature the greater the effect. The fact that the temperature immediately after the treatment had a similar effect was interpreted as a proof that the process of rejoining was affected rather than the production of breaks itself.

Catcheside *et al.* (2) have analysed the frequencies of chromosome aberrations produced in *Tradescantia* microspores by X-rays at 1, 20, and 30° C. Three kinds of aberrations were studied, *viz.*, chromatid breaks, isochromatid breaks and interchanges. The yield of chromatid breaks was found to be only slightly dependent upon the temperature at which the irradiation was made. The isochromatid breaks and interchanges on the other hand diminished markedly with increase of irradiation temperature. Sax (38) has confirmed these findings also in respect of the independence of the chromatid breaks of temperature.

When Thoday and Read discovered the oxygen effect, they assumed that "the effect of temperature observed by Sax and Enzmann and Catcheside *et al.* ... may, at least in part, be due to differences in the availability of oxygen" (42). Fabergé (5) has investigated the frequencies of chromosome breaks produced by X-rays in *Tradescantia* over the whole temperature range from 25° C to liquid air. He found that the sensitivity of the chromosomes shows a dip in the region of 0° C, and thereafter falls gradually, so that at liquid air temperatures the effect is about $^1/_5$ of that at 25° C. The curve for chromosome sensitivity at different temperatures resembles that of hydrogen peroxide production plotted against temperature when water containing oxygen is X-rayed.

The possibility, that the temperature effect may be due to the greater solubility of oxygen at low temperatures has been tested by Giles *et al.* (8) working with *Tradescantia* microspores. The amount of dissolved oxygen was varied in two different ways: either by varying the percentage of oxygen in the exposure chamber at a constant temperature, or by varying the temperature while the oxygen tension was kept constant. The results indicated, that the increased effect of X-rays at low temperatures cannot be attributed entirely to increased oxygen solubility.

Very interesting results were obtained by Giles *et al.* (8), when the irradiations were performed at different temperatures in nitrogen. A higher frequency of aberrations were obtained when the inflorescences were irradiated at high temperatures, which means that the temperature effect in the absence of oxygen is exactly opposite to that observed in the presence of oxygen.

It was found some years ago (24, 25) that the effect of EOC also is dependent on temperature during the treatment. As in the X-ray experiments described by Sax and Enzmann, an effect of temperature during the recovery period was also observed, but this effect was slight compared with that during treatment (24). In which way the temperature influences the chromosome aberrations produced by EOC appears from Fig. 4. The points are based on old data, which in part have been published previously (24). In these experiments the concentrations of EOC was 7.5×10^{-3} M and the period of treatment 6 hours. The root tips were fixed 1 day after the treatment.

The temperature influences the effect of EOC in much the same way as it influences the effect of X-rays. In fact, the only difference is that the maximum effect in the case of EOC is obtained at 10° C, while the chromosomes are most sensitive to X-rays in the region of 0° C. That the effect of EOC, in contrast to that of X-rays, decreases when the temperature is lowered below 10° C, could, however, be due to the fact, that penetration is rendered

more difficult. It is known that the permeability of biological membranes to organic compounds decreases rapidly with decreasing temperature. Therefore the influence of temperature can be added to the many similarities existing between the cytological effects of X-rays and EOC.

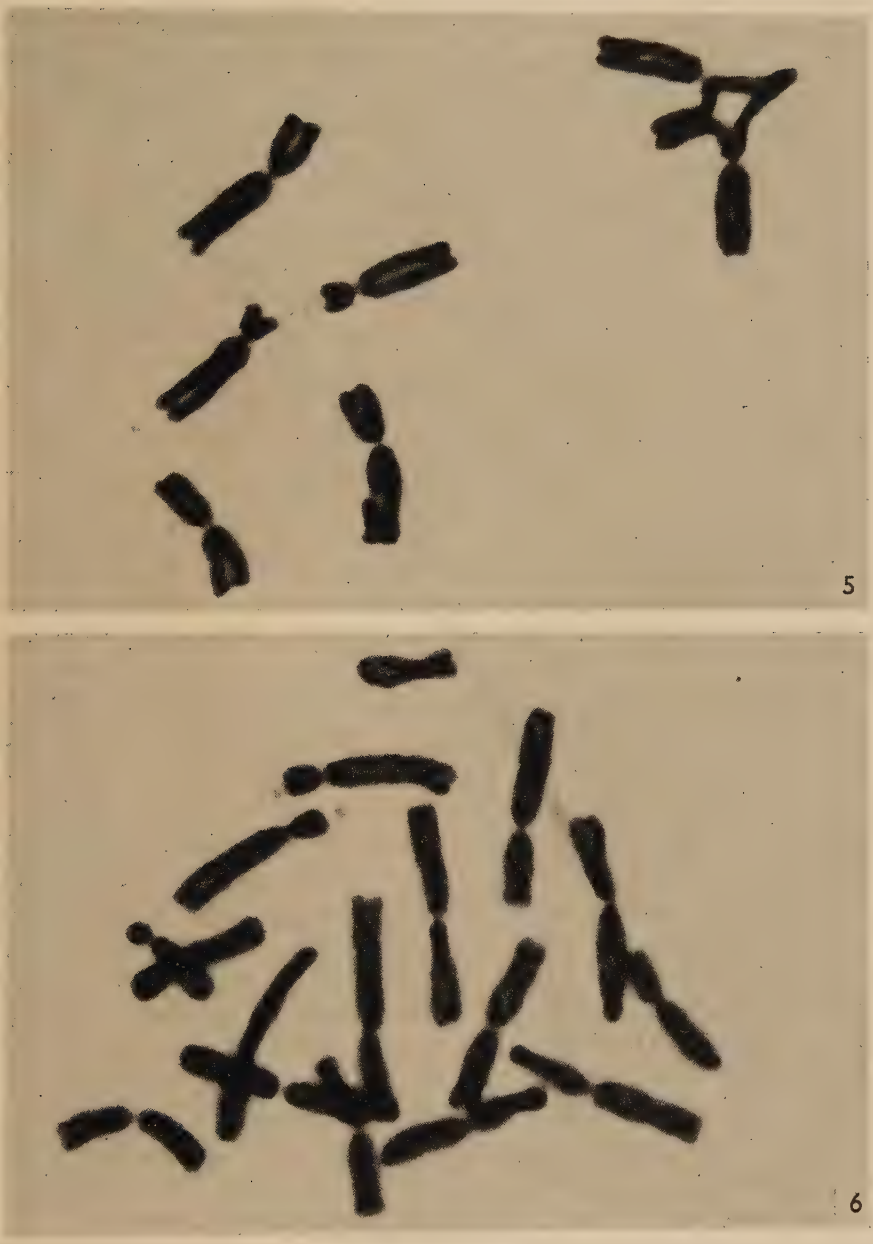
CONCLUSIONS

The experiments described above have shown that the effect of EOC on *Allium*, in contrast to that of other radiomimetic chemicals, imitates the effect of X-rays also in other respect than in the more general types of changes induced in chromosomes (physiological effects, structural chromosome changes). The interphase sensitivity to chromosome breakage by the two agents is thus very similar, and so are the relative frequencies of chromatid and isolocus breaks obtained various times after the treatments. Finally environmental factors, such as oxygen tension and temperature, seem to influence the production of stickiness and chromosome breakage by EOC and X-rays in a very similar manner. The only clearcut difference exists in the distribution of breaks, those produced by EOC occurring more frequently in the satellite fibers.

In regard to the mode of action of the purines, the results reported in the present paper do not support the suggestions of several authors, *e.g.* Biesele *et al.* (1), Kalckar, H. (20), that this is to be sought in the incorporation of the abnormal purines into nucleic acids. The chromosomes are most sensitive to purine derivatives at the very end of resting stage. But according to Howard and Pelc (19), the synthesis of deoxyribonucleic acid (DNA), as judged by the uptake of radioactive phosphorus, takes place in the middle of resting stage, when the effect of EOC is at a minimum. The DNA synthesis should therefore be finished when EOC is most active in producing chromosome breakage. Howard and Pelc point out, however, that the autoradiograph method used by them only show "the entry of phosphorus into newly formed DNA capable of withstanding acid hydrolysis, i.e. to some extent polymerized".

There are, however, other facts which contradict the metabolite-antimetabolite explanation of the effects of the radiomimetic purines:

1. No correlation exists between the ability to be incorporated into nucleic acids and the ability to produce chromosome breakage. 8-azaguanine and 2.6-diaminopurine are examples of such purines as can evidently be incorporated into nucleosides, nucleotides and nucleic acids (7, 18, 44, 30). Of these purines, 8-azaguanine does not produce chromosome breakage in



Figs. 5 and 6. The main types of structural chromosome changes produced by EOC in *Allium*. Fig. 5 shows a chromatid break, a chromatid interchange and breaks and reunion in the satellite fibers. Fig. 6 shows an isolocus break with sister union in both the centric and the acentric fragment. Feulgen squashes.

Allium, while 2.6-diaminopurine has not yet been tested. On the other hand, it seems very unlikely that the purines, which have a strong radiomimetic effect (e.g. caffeine and its 8-ethers and 8-thio-ethers, 1.3.7.9-tetramethyluric acid) could be incorporated, since they have no hydrogen atom at the 9 position, which could be substituted by ribose or deoxyribose.

2. The enzyme nucleoside phosphorylase, which catalyzes the synthesis of ribosides and deoxyribosides is not influenced by caffeine and its derivatives (Overgaard-Hansen and Kihlman, unpubl.).

3. The incorporation of radioactive adenine into adenosine-5-phosphate, catalyzed by pigeon liver homogenate, is not inhibited by the radiomimetic purines (Saffran, M., personal commun.).

4. The fact that the growth inhibiting and mutagenic effects of abnormal purines, in some cases can be at least partly reversed by the addition of natural purines such as adenine, hypoxanthine and their ribosides (22, 35) has been interpreted as a proof of the antimetabolite hypothesis. At the Institute of Physiological Botany in Upsala it was found some years ago, that the growth inhibiting effect of caffeine on the fungus *Neurospora crassa* could be antagonized by adenine or hypoxanthine. A similar antagonism was later found (Hultgren, Kihlman and Fries, under prep.) also in the fungus *Ophiostoma multiannulatum*. In this material, in addition to adenine, several other compounds were also tested for their ability to reverse the growth inhibiting effect of caffeine. It was found that tryptophan had an antagonistic effect of the same order as that of adenine, and a considerable antagonism was also obtained with procaine and aniline. Thus, since an antagonistic effect can be obtained also with structurally unrelated compounds, there is no reason to believe that the caffeine-adenine antagonism is dependent on the structural relationship between the two compounds, and therefore this antagonism is no proof of the antimetabolite hypothesis.

Because of the facts mentioned, it seems rather unlikely, that the production of chromosome breakage by purines is due to an effect on nucleic acid synthesis. The possibility that the purines act by interfering with the function of nucleic acids cannot yet be excluded, however. More experimental work is needed before any satisfactory explanation can be given to the radiomimetic action of the methylated oxypurines.

SUMMARY

The effect of 8-ethoxycaffeine (EOC) on root tip chromosome of *Allium cepa* was compared with that of 75 and 100 r of X-rays. The chromosomes

showed a very similar interphase sensitivity to breakage by the two agents, *i.e.* the maximum effect was obtained with EOC as well as with X-rays when the cells were treated at late interphase. The relative frequencies of chromatid and isolocus breaks different times after treatment were also remarkably similar in the two cases.

A marking technique for determination of the duration of the mitotic cycle and the sensitive stage to chromosome breakage was described. The marking was obtained by treating the cells with low doses of theophylline, which treatment resulted in the production of binucleate cells, but no other visible damage. The mitotic cycle as well as the interphase sensitivity was then studied in the binculeate cells.

The influence of oxygen tension on the radiomimetic effect of EOC was investigated. It was found that, as in the case of X-rays, oxygen increased both the stickiness effect and the frequency of chromosome breakage. In nitrogen the effect of EOC was between $^1/_3$ and $^1/_6$ of that in oxygen atmosphere.

In connection with the oxygen effect the influence of temperature on the production of chromosome aberrations by EOC and X-rays was reviewed.

I am greatly indebted to Dr. Arne Forssberg, the Institute of Radiophysics, Stockholm, for performing the X-ray irradiation. The investigation was supported by grants from Knut and Alice Wallenberg's Foundation and from the Swedish Cancer Society.

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Experimental Cell Research, **8**, *369–386 (1955)*

PEROXIDE AND ULTRAVIOLET RADIATION EFFECTS[1]

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Received July 15, 1954

Action spectra for the bactericidal effect of ultraviolet radiations (UV), retardation of division of cells, induction of mutations, activation of eggs, etc. suggest that UV radiations are absorbed by specific chemicals with absorption spectra corresponding to the respective action spectra (41, 42, 44). Such effects might be explained by direct action of the absorbed UV upon vital chemical constituents of the cell suggested by the action spectra. However, mutations and other changes were induced in bacteria placed in irradiated media. These effects must have been indirectly induced by diffusable photoproducts, since the organisms had not been exposed to UV (25, 26, 53, 87, 88, 97, 98, 99). The latter series of experiments suggest that possibly irradiation of the organism also produces similar photoproducts in the aqueous continuum of the protoplasm; therefore the seemingly direct effect of UV may really be indirect.

The substance produced by the irradiation of the medium or the aqueous continuum of the cell is conceived to be an oxidizing radical or some sort of peroxide, since oxygen is liberated by catalase from irradiated media (28, 98, 99). Earlier colorimetric determinations had suggested the presence of peroxide in irradiated media (11, 60). While hydrogen peroxide is detected in irradiated organic media, it is believed to be in equilibrium with organic peroxides (28, 99). It must not be forgotten that injurious but unidentified substances other than peroxides may also be formed by irradiation of media (10, 13, 15).

If the effects of UV are due to peroxide formation, then a similarity might be expected between the action of peroxide and these radiations. The effects of peroxide and UV used jointly should also be additive. Furthermore, catalase should protect organisms from UV as it does from peroxides; non-

[1] Supported in part by funds made available on US Public Health Grant C-1799 (C2). The authors are also indebted to Mr. Frederic Falconer, head librarian, Biological Libraries, Stanford University, for generous help in bibliographic research, to Messrs. Dudley Golkin and Garth Miller for preliminary experiments on effects of hydrogen peroxide on proteins and nucleic acids and to Mr. C. L. Brandt for preliminary experiments with catalase.

toxic reducing agents should have a similar effect. A comparison was therefore made between the action of hydrogen peroxide and UV upon protozoans, gametes of the sea urchin and upon the absorption spectra of some of the major chemical constituents of cells.

MATERIALS AND METHODS

The protozoans, grown according to methods previously described (40, 48), were exposed to dilutions of hydrogen peroxide (Superoxol) made up in protozoan balanced salt medium.

Marine ova were treated in the manner described elsewhere (36). They were irradiated and in some cases photoreactivated in the manner already described (45, 96). Monochromatic UV (2654 Å) and visible (4350 Å, blue) measured with a calibrated thermopile were used in the experiments reported. UV treated cells were kept in the dark and observed with red lights. Catalase from a crude beef liver extract was used in preliminary trials but crystalline catalase (Armour; Worthington) was used in definitive experiments. For measurement of the absorption spectra the various compounds were dissolved in M/20 sodium phosphate buffer at a pH of 7.0–7.2. Absorption was determined with a Beckman DU spectrophotometer in the manner previously described (47).

EXPERIMENTAL RESULTS

Differential resistance of protozoans and sea urchin larvae to hydrogen peroxide and UV. The protozoans at first showed little reaction to the hydrogen peroxide to which they were added with the exception of *Euplotes sp.* and *Paramecium bursaria* which became more active. In all species locomotion slowed visibly within a short time after exposure. Most of the protozoans swelled as the contractile vacuoles became inactive. Cytolysis followed excessive uptake of water, even in *Condylostoma magnum*, a marine form. However, *Euglena viridis* rounded up without swelling.

Each of the protozoans cytolyzes in a characteristic way. *Blepharisma undulans* begins to cytolyze at the anterior end; once the cell has ruptured the internal contents pour out through the opening. *Fabrea salina* cytolyzes from the cytopyge anteriorly. *Paramecium caudatum* swells until the oral groove is obliterated and the trichocysts are discharged, when it suddenly disintegrates. Cytolysis of *Didinium nastutum, Colpidium colpoda* and *Tillina magna* is similar to that of *Paramecium*. The pelicle of *Condylostoma* and *Amoeba proteus* ruptures at random, followed by cytolysis. When *Amoeba* cytolyzes locally, the injury is often repaired and streaming continues inside. After a series of such injuries, streaming ceased and the amoebae died and

cytolyzed. However, the events preceding death and the manner of cytolysis of various protozoans is to some extent dependent upon the concentration of peroxide. In a high concentration, for example, 0.3 per cent, the protozoans seem to be coagulated without cytolysis.

The time required for immobilization of the different protozoans in 0.0088 M H_2O_2 increased in the order given below:

Amoeba, Paramecium bursaria, Colpidium colpoda, Stylonychia curvata, Blepharisma, Tillina, Tetrahymena, Paramecium caudatum, Euplotes, Condylostoma, Euglena, and *Fabrea.* Most amoebae died within two minutes, while the fabreas died within thirty.

Exposing paramecia to 1:10,000 catalase for 15 minutes then centrifuging them and rinsing four times, protected paramecia against H_2O_2 to the extent that they endured twice as long in 8.8×10^{-2}M, 8.8×10^{-3}M and 8.8×10^{-4}M H_2O_2. This suggests that catalase is adsorbed to the protozoans, or engulfed and therefore serves to protect them. On the other hand rinsing does not remove all the catalase in solution therefore part of the effect may be due to the catalase in solution again diluted to the extent of about 1:10,000 or more by the washes.

The blastulae (30 hours after fertilization, kept in a constant room temperature at 13° C) and gastrulae (48 hours old) of the sea urchin, *Strongylocentrotus purpuratus,* were inactivated in about five minutes in 8.8×10^{-2}, 8.8×10^{-3} and 8.8×10^{-4}M H_2O_2; they died after about an hour in 8.8×10^{-5} M and 8.8×10^{-6}M but lived indefinitely in 8.8×10^{-7}M. In comparison paramecia lived indefinetely in 8.8×10^{-6}M. The multicellular state of the sea urchin larvae does not render them more resistant to peroxide than the paramecia.

Effect of hydrogen peroxide on division rate of Colpidium colpoda. Colpidia which had been exposed to a solution of hydrogen peroxide until they had slowed down and begun to swell, were placed in freshly bacterized culture medium and observed from time to time for division. As shown in Fig. 1 A the treated colpidia divide at the same rate as controls and division starts almost the same time. Exposure to hydrogen peroxide is therefore harmless provided the animals have not been injured to the point of cytolysis. The early stages of peroxide injury are therefore reversible and do not affect even so delicate a process as cell division. However, once treated colpidia have begun to swell and cytolyze, they invariably die. In contrast colpidia exposed to UV radiation are affected in proportion to dosage (Fig. 1 A).

The effect of UV and peroxide might perhaps be more effectively compared by treating colpidia with both agents in sequence. If UV produces peroxide

in the animal, addition of peroxide after irradiation should exaggerate the effects. This was tried with *Colpidium* and the results are also shown in Fig. 1 A. Exposure to peroxide for a time less than that required to cytolyze does not significantly increase or decrease the effectiveness of UV in retarding division of colpidia.

The effect of peroxide on sea urchin sperm and eggs. Although sperm of the sea urchin are somewhat more sensitive than eggs, peroxide inactivates both. Eggs treated for five minutes with 8.8×10^{-2}M H_2O_2 developed fertilization membranes when later inseminated whereas most sperm exposed for five minutes to even 1/10 or 1/100 this concentration were unable to fertilize eggs. Whether sperm or eggs were treated, relatively normal development, as judged by cleavage and the formation of blastulae and gastrulae, occurred only when the concentration of peroxide was 8.8×10^{-5}M or less. Fertilized eggs showed a similar susceptibility, indicating that peroxide affects the cells, not just the reactions involved in fertilization.

When sperm and eggs are first treated for five minutes with catalase (usually in low concentration of 1:100,000 or 1:80,000 but in a few cases with as high a concentration as 1:1000), development usually occurs in ten times more concentrated peroxide solutions than in absence of catalase. Catalase protects sperm from peroxide more effectively than eggs. The experiments do not tell whether the catalase is absorbed or adsorbed and acting superficially. Furthermore it is always likely that in spite of washes, some catalase is also present in solutions and protects against peroxide by reducing the concentrations outside the cell.

Although eggs treated with low concentrations of peroxide develop, cleavage is somewhat retarded. Therefore, while the effect is not all or none, it is graded over a very narrow concentration range, in contrast to the gradually graded effects of UV (36).

Tests for protective effect of catalase upon UV treated protozoans. Preliminary studies indicated that catalase was toxic to colpidia. While colpidia placed in crude liver catalase solutions swam, ate the particles present and even divided, the beginning of division was generally retarded and the rate of division was decreased. Concentrated catalase may be toxic because it acts as a peroxidase catalyzing oxidations which interfere with cell division. It is unlikely that an extraneous poison was the cause of toxicity since the sample of crystalline catalase (minimum activity equals 250 units per c.c.) used in the concluding series of experiments (Fig. 1 B and 2 A) was prepared by methods insuring absence of heavy metals and non-volatile solvents (courtesy of Dr. Robbins of the Armour laboratories). The catalase used

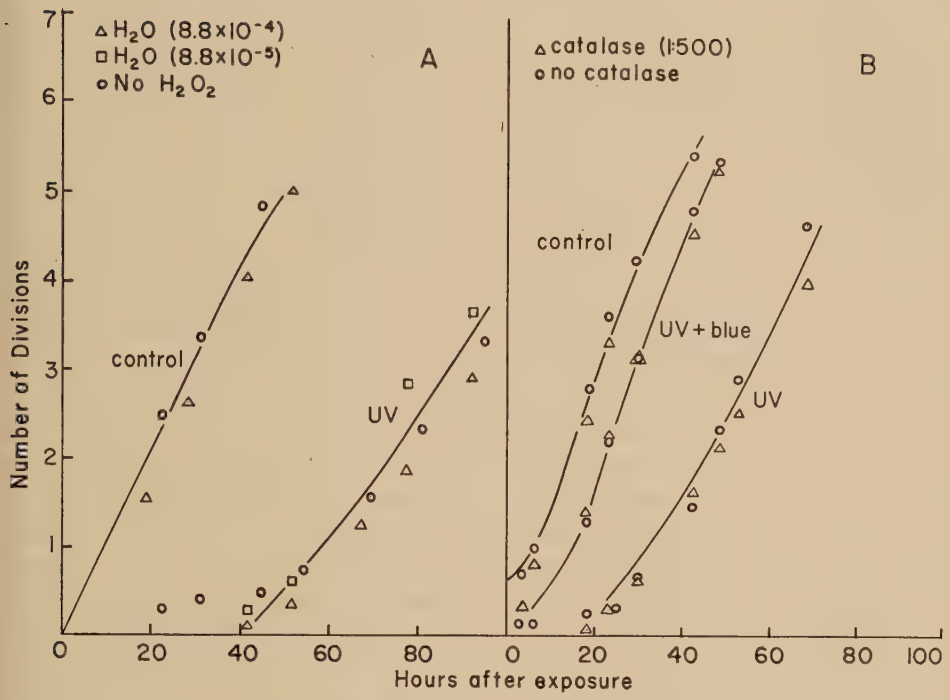

Fig. 1 A. Note the retardation of division by UV (dose of 1000 ergs/mm² at 2654 Å) but the slight effect of peroxide (10^{-4} M). Prolonged sojourn of colpidia in peroxide of this concentration is lethal. Peroxide treatment before UV does not affect the effectiveness of the UV.
Fig. 1 B. Note that photoreversal by blue light (dose of 57,000 ergs/mm² at 4350 Å) of UV injury (1000 ergs/mm² at 2654 Å) occurs equally well in presence or absence of catalase and that catalase alone does not reverse UV injury. The control is not affected by catalase.

in radiation experiments was diluted until it did not retard division of colpidia (1:100 to 1:2560, crystalline catalase).

Since treatment with catalase may protect against exposure to UV only if applied at a critical time, colpidia were treated with catalase in a variety of ways. Some were exposed for 2 to 24 hours before irradiation to enable them to take up the catalase, some only during irradiation, others for several hours after irradiation, and still others before and during irradiation or during irradiation and immediately thereafter (Fig. 1 B).

Although the preliminary experiment suggested it, no evidence of protection of colpidia from UV injury or reactivation by catalase was obtained, in eight series of experiments, even when the control with catalase was comparable to the control without it. The UV treated colpidia in many cases were injured by treatment with catalase subsequent to UV. Furthermore,

the injurious effects of catalase on UV treated colpidia were cumulative and became more pronounced as division proceeded in the catalase solution.

Treatment with catalase does not improve photoreactivation by blue light, since equal photoreactivation occurs in the presence and in the absence of catalase (Fig. 1 B). Since the same degree of photoreactivation by blue light occurs in the presence of catalase, injury resulting from catalase must be different from that produced by UV, only the latter being reversed.

Because starved colpidia are very much more sensitive to UV injury (46), it seemed possible that they might also serve as more sensitive indicators for testing catalase reactivation of UV injury. However, the results of three series of experiments were in no way different from those obtained with fed animals.

When paramecia (*Paramecium multimicronucleatum*) were treated with crystalline catalase (1:1250) and then fed to irradiated didinia (*Didinium nasutum*, a carnivorous protozoan), no evidence of reversal of the injurious effect of UV on the didinia was observed. In this case the *Didinium* engulfed the paramecia, therefore any catalase adsorbed or ingested by the paramecia is taken internally by *Didinium* and should be available within the cell. The effect of treatment upon the time required to reach the fourth division was taken as a measure of its usefulness. Not only did didinia not benefit but they were injured, the fourth division after treatment with both catalase and UV being delayed more than after UV treatment alone.

Since the ecology of colpidia, didinia, etc. is rather complex, tests for catalase reactivation of UV injury by concomitant or subsequent exposure to catalase were made on *Tetrahymena geleii*, a protozoan which can be grown in sterile culture in the absence of any other organisms. No evidence for reversal of UV injury by pretreatment with catalase was obtained. However, photoreactivation occurs readily in *Tetrahymena* (24 a).

Test for catalase protection with sea urchin sperm. Since catalase may fail to protect protozoans because of its inability to get inside the cell where the UV may have its effect, a series of experiments were tried with sea urchin sperm, since the sperm is almost a naked nucleus and extremely sensitive to UV (37).

The spawn was diluted 1:200 with sea water containing 0.05 M glycine, then an equal volume of catalase in sea water was added in one case and boiled catalase of the same concentration to the control. Crystalline catalase in the range of 1:1000, 1:10,000 and 1:100,000 was used in the experiments. Both samples were irradiated with UV of wave length 2654Å, a dose of 50, 100, 200 or 1000 ergs/mm^2 being used, these dosages being known to retard

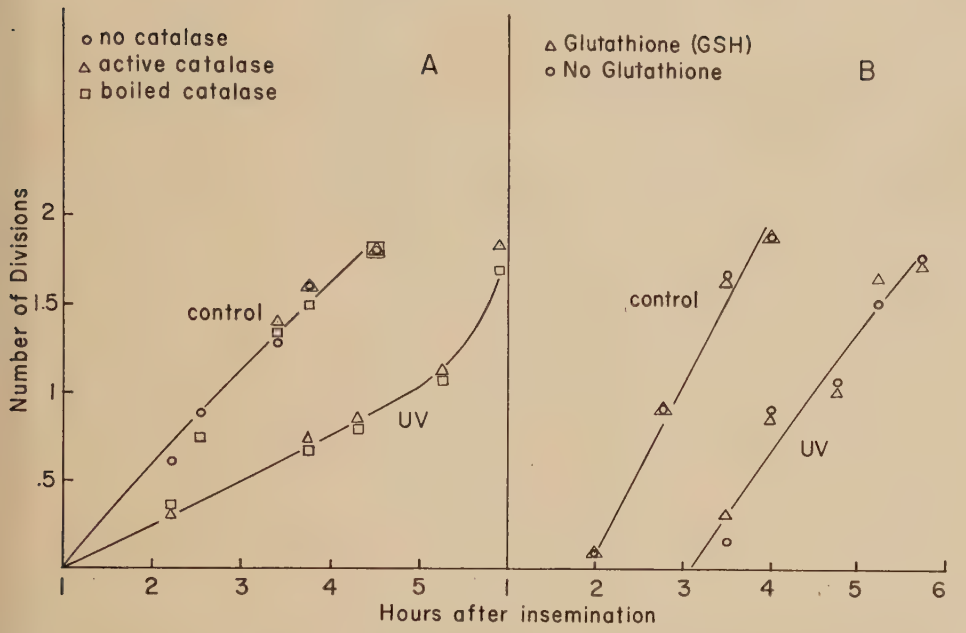

Fig. 2. The retardation of division of eggs of the purple sea urchin fertilized by sperm irradiated with a dosage of 100 ergs/mm² short UV (2654 Å) is compared in A in the presence of active and autoclaved catalase (concentration of 1:1000) and in B in presence and absence of glutathione (10^{-3} M) during irradiation.

division of eggs fertilized with the sperm (37). The effect of the radiations was followed by the rate of cleavage and formation of embryos of the treated sperm used to fertilize untreated eggs. For a few hours after insemination, observations were made at 15 to 30-minute intervals and the number of embryos present as 2, 4 celled stages etc. was counted. In four series of experiments, no decisive evidence for reversal of UV effects was observed. Possibly a slight advantage could be detected in a few cases (Fig. 2A).

The effect of glutathione on ultraviolet action. If peroxide is formed by UV, a reducing agent should reverse the injury if it is present at the locus where the oxidizing agent is formed and at the time of its formation. Cysteine proved quite toxic to colpidia, but glutathione (10^{-4}M) was not. Colpidia starved for 48 hours in balanced salt solution were used for the experiments because they are a more sensitive indicator of the effects of UV radiations (46). When irradiated in 10^{-4}M glutathione, generally they were found to be less injured than when irradiated in the balanced salt solution alone. Occasionally they seemed to be slightly more sensitive to UV. The beneficial effect could

TABLE I

Protection from ultraviolet (UV) injury by glutathione (GSH).[1]

Experiment	Hours to third division		Delay UV	Per cent recovery GSH + UV	Per cent recovery UV + GSH + Vis.[3]	Per cent recovery UV + Vis.	Per cent recovery GSH + UV + Vis.
	Control	UV[2]					
1	30	102	72	16	—	55	55
2	29	111	82	27	—	60	64
				21			
3	27	98	71	−11	52	52	53
4	27	127	100	10	61	53	61
5	32	115	83	−7	46	46	53
6	31	119	88	22	—	63	—
7	28	75	47	5	—	—	—
8	31	71	40	44	—	—	—
9	30	95	65	46	—	77	—
10	29	93	64	38	—	38	—
11	30	84	54	10	—	—	—

[1] Glutathione in balanced salt solution, 10^{-4} M.
[2] Dosage of UV (2654 A) always 400 ergs/mm².
[3] Dosage of blue (4350 A), 194,400 ergs/mm².

be due to physical screening, but measurement of the absorption by solutions used indicated that at most only a few per cent of the radiations were absorbed by a layer 5 mm thick, whereas only one mm of the solution was used in the experiments related.

Of greater concern is the occasional deleterious effect of glutathione (Table I). In preliminary experiments glutathione was found to be injurious especially after prolonged exposures. If exposure to glutathione is made after UV treatment, the effects are occasionally deleterious as well. It is possible that the enzyme systems in the cell concerned with cell division hover in a precarious zone from which they may be displaced one way or the other depending on a nice balance which our attempts at control do not disclose. Since glutathione is readily oxidized, a product toxic to irradiated cells might have been produced. However, aerated glutathione protected from UV injury to the same extent as a sample treated with nitrogen for the same length of time (Table I). Since our experience with the protozoans is extensive, the variations are less readily attributed to clumsiness in handling. The controls divide with fairly consistent regularity, as do those exposed to UV (Table I).

TABLE II

Changes in density of various compounds after exposure to hydrogen peroxide.

Substance	Control + 0.02 cc Catalase				H_2O_2 + 0.02 cc Catalase				
	Conc. in g/1	Wave length in Å			Conc. in Mols.	Dosage in min.	Wave length in Å		
		2400	2800	3100			2400	2800	3100
Serum	1	.626	.371	.030	.38	60	.571	.337	.020
Albumin		.641	.379	.031		360	.581	.342	.020
Aliphatic amino acids	5.6	.520	.262	.122	.22	30	.452	.233	.078
Tyrosine	.342	.542	1.200	.097	.44	30	.560	1.202	.101
Phenyl alanine	.225	.242	.173	.090	.44	30	.366	.229	.114
Tryptophane	0.20	.259	.658	.032	.35	30	.307	.769	.137
	Conc. in mg/1	2300	2600	2900			2300	2600	2900
RNA	20	.168	.282	.063	.35	30	.150	.262	.056
DNA	20	.187	.229	.068	.35	30	.256	.362	.220
Adenine	7.5	.169	.394	.033	.35	30	.236	.423	.094
Uracil	10	.161	.331	0.28	.35	30	.204	.376	.089
Adenylic acid	15	.150	.360	.030	.35	30	.230	.469	.152
Uridylic acid	20	.146	.277	.041	.35	30	.144	.267	.030
Adenosine	10	.154	.300	.031	.35	30	.172	.285	.025
Uridine	20	.172	.401	.039	.35	30	.192	.422	.046

The results of treatment with glutathione might be taken as evidence in support of the peroxide theory of UV action, although by no means conclusive.

Although glutathione reverses UV injury in many cases, it is seldom as effective as visible light. When it is effective, its action is generally not additive to that of visible light (Table I). This would suggest that the effect of the two agents is similar. The irregularity of the experiments makes a decisive answer impossible at this point.

Because the sperm presents a nucleus so exposed to UV as compared with the egg or a protozoan, attempts were made to protect the sperm of the sea urchin from UV by glutathione. Sperm immersed in the highest concentration of glutathione not injurious to controls (10^{-3} M) were irradiated with a dosage of 100 ergs/mm^2 and used to inseminate unexposed eggs. The eggs kept at 13° C in a constant temperature room were observed with red

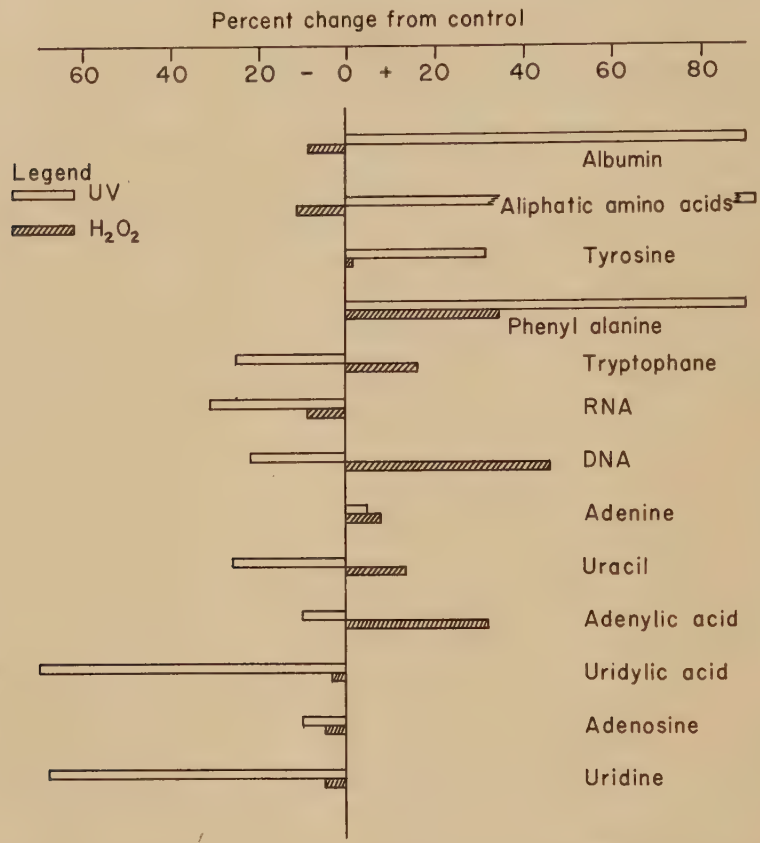

Fig. 3. Histogram made from representative single experiments comparing the percentage change in absorption at 2800 Å (near maximal) for serum albumin, aromatic and aliphatic amino acids, and at 2600 Å for nucleic acids, nucleotides, nucleosides, purines and pyrimidines treated with UV and H_2O_2. The variability is not indicated but small changes cannot be considered significant. Treatment 30 minutes with .35 M H_2O_2 except for serum albumin (.38 M), aliphatic amino acids (.22 M), tyrosine (.44 M) and phenyl alanine (.44 M). A 0.02 cc sample of crystalline catalase (Worthington, 250 units/cc) was always added to 20 cc samples of control and peroxide-treated compounds to release the H_2O_2.
Exposure to UV from sterilamp (mainly 2537 Å), 4 hours for serum albumin, aliphatic amino acids, DNA, adenine, uracil, adenylic acid and adenosine; 3 hours for tyrosine, tryptophane, and RNA; 1 hour 38 min. for phenyl alanine and 1 hour for uridylic acid and uridine. UV intensity 35 to 45 ergs/mm². The area of the quartz-covered cell was 18.9 sq. cm, the depth of the solution was about 8 mm and the solution was magnetically stirred during exposure.
The broken line for the aliphatic amino acids indicates a greater change (300 per cent) than can be put on the graph.

light from time to time for cleavage and the per cent cleaving was determined. The data plotted in Fig. 2B indicate no protection from UV injury.

The effects of hydrogen peroxide on the absorption spectra of nucleic acid

and proteins. The effects of peroxide on nucleic acids, representative nucleotides, nucleosides, purines, and pyrimidines on albumin and representative amino acids are summarized in Table II.

Brief exposure to peroxide of such low concentration as might possibly result from action of radiations, affects the absorption spectrum of most compounds little if at all. Exposure of desoxyribonucleic acid to concentrations of hydrogen peroxide much higher than could be expected from radiation decreases the absorption of UV over the entire span studied. The effectiveness of the treatment increases with the concentration of peroxide and the length of exposure, in these respects resembling the action of UV (64). Similar conclusions may be drawn for ribonucleic acid. In many cases exposure of compounds to high concentrations of peroxide changes absorption in reverse of that caused by UV. For example, the absorption of UV by serum albumin after long exposures to higher concentrations of hydrogen peroxide (see 9) progressively declines over the entire spectrum (Table II). In contrast the absorption of UV by serum albumin increases all along the spectrum following UV irradiation (30, 47). Data for albumin and for various compounds not previously investigated are given for several wave lengths in Table II.[1] In Fig. 3 the data for increases or decreases caused by peroxide or UV at the 2800Å maximum are given for amino acids and albumin and for the 2600Å maximum for nucleic acids and their derivatives. Even a glance at the figure indicates how different the effects of the two agents are in many cases.

DISCUSSION

The purpose of this paper was primarily to ascertain whether the effects of UV upon cells could be interpreted in terms of the formation of peroxide (97). The data of the present study and the data in the literature on the effects of peroxide and UV upon viability, mutations and various activities of cells are examined below for supporting or contradictory evidence (see also 3a and 32a).

Extensive studies of the effect of UV on viability of various bacteria and

[1] The addition of hydrogen peroxide to serum albumin results in an increase in absorption far greater than the sum of the absorption of the peroxide and the albumin. This suggests that the two compounds have reacted to form a complex absorbing more than the sum of the absorption of each of the two constituents taken separately. Consequently, a 0.02 cc sample of crystalline catalase (Worthington, 250 units/cc) was always added to a 15 cc sample of the control and peroxide-treated compounds to decompose the peroxide and its complexes. The changes in absorption recorded in Table II are therefore the changes effected by peroxide alone. The data are given for representative experiments and do not include a measure of the variability. Small changes cannot be considered significant.

fungi have appeared (see 29). While a number of studies have also been made upon the comparative sensitivity of some bacteria and fungi to peroxide, e.g. *Staphylococcus aureus* is retarded by 8.8×10^{-4}M and killed by 1.07×10^{-2}M (83 see also 65), yeast is retarded by 6.9 to 8×10^{-3}M (100), and *Penicillium glaucum* will grow in 1.7×10^{-2}M but is inhibited by 8.8×10^{-2}M (77), a significant comparison with UV action cannot be made because the organisms used are not comparable to those in which the effect of UV has been determined. The most telling data for comparison of action of UV and peroxide would be for a series of bacteria with different catalase content, a decreasing susceptibility to UV being expected with increased catalase content if UV acts by production of peroxide. Comparison between the effects of UV (35) and peroxide upon the viability of various protozoans of the same species, presented in this paper indicates that the susceptibility of the protozoans to the two agents is not the same.[1]

The changes in appearance of protozoans subjected to UV (38) or to peroxide (this paper) such as slowing, enlargement, cessation of vacuolar movement and cytolysis are rather similar. The only difference between the action of the two agents is the more rapid swimming which usually follows exposure to UV, a change not often seen on immersing the protozoans in peroxide. Several early papers describe effects of peroxide on *Amoeba* (32) and on trichomonads (66).

Both UV and hydrogen peroxide are capable of producing mutations in bacteria (97) and in *Neurospora* (28). In the former case, hydrogen peroxide is effective only in organic media whereas in the latter case it is also effective even in inorganic media. However, the relative effectiveness of UV and peroxide on production of mutations in a number of organisms, has not been determined.

UV and hydrogen peroxide affect division of cells in different ways. UV retards division of *Colpidium* in proportion to dosage (Fig. 1). Hydrogen peroxide either has no effect on division or kills the colpidia.

The respiration of *Arbacia* sperm is inhibited by 0.001 M hydrogen peroxide but is stimulated by one tenth this concentration (7, 8); respiration of mouse skin is also stimulated at low concentrations of peroxide and inhibited at high (23, 69). Respiration of brain tissue (68a) and cancer tissue (89) is decreased by peroxide although in the latter, glycolysis and growth are not changed. The Pasteur effect in erythrocytes is suppressed by

[1] Decreasing susceptibility to H_2O_2: *Amoeba, Paramecium bursaria, Colpidium, Stylonychia, Blepharisma, Tillina, Tetrahymena, P. caudatum, Euplotes, Condylostoma, Euglena* and *Fabrea.* Decreasing susceptibility to UV: *Blepharisma, Stylonychia, P. caudatum, P. bursaria, Amoeba, Colpidium, Tetrahymena, Euglena* and *Fabrea.*

peroxide (84). UV also stimulates the endogenous respiration of yeast, but it decreases the exogenous respiration of yeast (39) and of luminous bacteria (38) although the effect is not at once evident. Because the studies with peroxide and UV have not been made on the same organisms a comparison significant for determining similarity or difference in action cannot be made.

If UV damage to cells results from peroxide formation, addition of catalysts such as catalase or ferrous iron which decompose peroxide should protect cells against UV. Catalase has been found to protect lysogenic bacteria against UV injury whether added before or after exposure to UV (62, 71). Production of mutations by UV in *Escherichia coli* is reduced by presence of catalase or ferrous salts in the medium (98). However, catalase failed to protect protozoans or sea urchin sperm against UV damage (this paper). It is possible that catalase fails to protect protozoa and sperm against UV injury because it does not enter into the cells or into that part of the cell which is most sensitive to UV, e.g. inside the nucleus (22).

If UV forms peroxide, addition of substances which reduce peroxide might also protect cells against UV. Such results have been reported for bacteria (62, 78, 93). Glutathione, of a concentration with negligible UV absorption was found to protect the protozoan *Colpidium colpoda* (Table I) against UV in experiments reported in this paper. However, the sperm of the sea urchin were not protected. Respiratory poisons such as carbon monoxide or cyanide in concentrations which inhibit respiration, protect *E. coli* against UV (12, 70), but do not protect sea urchin sperm (this paper). Some metabolic poisons alone increase mutation, perhaps by causing accumulation of peroxide. Sulfhydryl poisons also increase mutation (26) suggesting that sulfhydryls are the seat of action of oxidizing agents.

Conditions favorable for the accumulation of peroxide should increase the effectiveness of UV if the latter acts by peroxide production. Although UV kills even in the complete absence of oxygen ((13, 95) also Iverson and Sanders, unpublished), no studies have yet been made to determine its action at different oxygen tensions, although extensive studies of this type have been made with x-rays (2, 4, 5, 6, 49, 50, 54, 55, 56, 86, 92). X-ray action has even been considered as similar to oxygen poisoning (34a). Preliminary studies in this laboratory indicate no increase in action of UV on viability of yeast at different oxygen tensions. However, Kaplan (57) found that UV-induced mutation was facilitated in *Serratia marcescens* by increased oxygen tension. Other means of increasing peroxide concentration in cells exist, e.g., by use of various bacteriostatic agents (68, 83), but the effect of increase in internal peroxide on resistance of the organism to UV has not been

studied, although it should prove interesting. Catalase negative bacteria are more susceptible to UV than catalase positive bacteria, suggesting a protective effect of catalase (74), but they are not protected by addition of catalase (56a). However, other enzymes capable of decomposing peroxide are thought to exist (52).

If UV acts on cells by production of peroxide then peroxide and UV should produce similar effects on the major constituents of cells such as nucleic acids, proteins and their components (3). Although high concentrations of hydrogen peroxide, unlikely to be formed by UV treatment of the cell, decrease the absorption of UV by DNA and affect absorption spectra of purines, primidines, nucleosides and nucleotides, low concentrations such as might possibly appear after irradiation have no effect. UV depolymerizes nucleic acids (51), while peroxide does not (21, 27, 90, 91). Peroxide decreases the absorption of UV by serum albumin whereas UV increases it at the 2800Å maximum. Peroxide like UV alters the scattering properties of protein, suggesting changes in aggregation (79, 80, 81, 82), but has no effect on the electrophoretic pattern whereas UV exposure flattens the peak indicating a change in the molecule of egg albumin (19). Peroxide like UV reduces the viscosity of collagen solutions (79, 80). Unlike UV which hydrolyzes proteins in solution (43) or in monolayers (72, 73) peroxide has slight activity. Both radiations and peroxide alter the serological properties of tobacco mosaic virus (85). In conclusion, it must be admitted that UV and peroxide are generally quite different in action on cell constituents. However, this could mean a different end result of more drastic treatment than that required to stay important biological functions.

Peroxide is known to affect the nutritional value of some vitamins, such as riboflavin (61), nicotinic acid (17) and biotin (76), and to decrease the activity of such enzymes as proteinase (14), lipase (96) and sucrase (33). Radiations have similar effects upon enzyme systems (67). The two agents may therefore injure cells by their action on enzymes and other biocatalysts. However, sensitivity of enzymes inside and outside the cell is quite different (56b). No studies appear to have been made on the effect of peroxide on nucleic acid synthesis although changes occur after UV (59).

Recently the suggestion has been made that the photoreactivation of UV injured cells may result from decomposition by visible light of peroxide formed by action of UV on the cell (75), because catalase reactivation of some bacteria is increased by exposure to visible light (62, 75). Furthermore, a medium which has developed a mutagenic factor (peroxide?) after UV exposure loses some of its potency after treatment with visible light (53).

In addition, when the catalase content of the bacteria is high, photoreactivation is more likely to occur (C. Beam, personal communication (18)). Since catalase absorbs little visible light, active decomposition of peroxide by visible light would probably have to be accomplished by some light absorbing material combined to catalase. However, if visible light decomposes radiation-induced peroxide, it should protect cells aginst X-rays since action of these radiations is thought to be due largely to peroxide. However, X-ray damages are not photoreactivated (16, 24, 31, 61, see also 1). The correlation between photoreactivation and peroxide decomposition is therefore not good.

The action spectra for various UV effects on cells are difficult to interpret on the basis of indirect action of UV such as by the formation of peroxide, because these action spectra cover the range 2000–3000Å, whereas production of peroxide in exposed organic media has been observed only when wave lengths shorter than 2145Å are used (53). Water absorbs only wave lengths shorter than 1750Å (34). One could argue that such a short wave length limit was observed by the above workers because the media used did not contain compounds which could absorb at wave lengths longer than 2145Å. One might also argue that in the cell absorption of UV would produce a peroxide of nucleic acid or some vital protein. However, an irradiated mixture of nucleic acid was not mutagenic (58). In a sense, also, this interpretation begs the question since it is then a direct effect of irradiation.

From the evidence presented it would appear that while many striking similarities in action of peroxide and UV exist and that variation of the environment in many cases increases or decreases the effectiveness of UV on organisms as could be predicted by the effects on the concentration of peroxide formed by UV, quite a few effects of UV are not readily explained by the peroxide theory and then some are even contradictory to it. The possibility that UV injury is due to the formation of an oxidizing free radical or a peroxide can only be proved or eliminated by accumulation of more critical data from experiments some of which are suggested in the present survey.

SUMMARY

1. The action of ultraviolet radiations (UV) and peroxide are compared on viability and division rate of protozoans and sea urchin gametes and on common chemical constituents of cells. The literature pertinent to interpretation of UV action by peroxide formation is briefly reviewed.

2. The order of susceptibility of a number of protozoans to UV and hydrogen peroxide is compared. A different order is observed for the two agents.

3. Catalase fails to protect *Colpidium colpoda* from UV damage although it protects protozoans from peroxide externally applied. Catalase also fails to protect sperm of the sea urchin, *Strongylocentrotus purpuratus*, from UV injury as judged by the rate of division of untreated eggs fertilized with the treated sperm. Catalase (crystalline) in high concentrations is toxic to protozoa and gametes of the sea urchin.

4. Over a narrow concentration range hydrogen peroxide has a graded effect upon division of colpidia and eggs of the sea urchin. In contrast UV has a graded effect over a wide range.

5. Peroxide does not further injure UV treated colpidia as judged by division rate.

6. UV and peroxide often have opposite action on the absorption spectra of cell constituents such as proteins, nucleic acids, nucleotides, etc.

7. Enough difference in action between UV and peroxide exists to cast doubt on interpreting UV action by peroxide formation. Enough similarity exists in their action on some biological phenomena to make further pointed experiments desirable.

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Experimental Cell Research, **8**, *387–393 (1955)*

GRUNDLAGEN DER PHOTOMETRIE MIT WECHSELLICHT[1]

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Eingegangen am 25. Juli, 1954

Über die Bedeutung der Spektralphotometrie für die mikroskopische Forschung braucht an dieser Stelle nichts gesagt zu werden. Aus den grundlegenden Untersuchungen von Caspersson (3) wissen wir, daß sich mit Hilfe der mikroskopischen Spektralphotometrie die beiden großen Stoffgruppen der Eiweiße und der Polynucleotide, die für den Chemismus und den Stoffwechsel der Zelle von ganz grundsätzlicher Bedeutung sind, daneben aber auch verschiedene andere chemische Körper innerhalb einer Zelle mit hinreichender Genauigkeit nachweisen lassen.

In jedem Fall erhält man als erstes praktisches Ergebnis eines derartigen Analysenganges bekanntlich Absorptionskurven der verschiedensten Art. Die Registrierung dieser Absorptionskurven geschieht meist von Hand. Das heißt, daß ein Beobachter notwendig ist, um das Meßinstrument auf die gewünschte Wellenlänge einzustellen und um die einzelnen Meßwerte abzulesen und zu registrieren. Neuerdings sind auch vollautomatische Spektralphotometer konstruiert worden, die diese Funktion des Beobachters überflüssig machen. So haben zum Beispiel Caspersson (3, 4) und Thorell (10) über selbstregistrierende Mikrospektralphotometer berichtet, bei denen die Wellenlängenverstellung des Monochromators mit dem Vorschub des Registrierpapiers gekoppelt ist, auf dem dann unmittelbar das Absorptionsdiagramm erscheint. Eine einzelne Messung, d.h. *ein* Durchgang durch den in Frage kommenden Teil des Spektrums, erfordert dabei eine gewisse Zeit, die sich in der Größenordnung von einigen Minuten, mindestens aber mehreren Sekunden bewegen dürfte. Dieser Zeitraum ist aus apparativen Gründen nicht einfach zu unterschreiten.

Bestimmte Probleme, beispielsweise die Frage nach dem Einfluß der UV-Bestrahlung auf das UV-Absorptionsvermögen von Eiweißkörpern, erfordern aber eine weitere Verkürzung dieser Durchgangszeit. Das ist insbesondere darum von grundsätzlichem Interesse, weil in letzter Zeit, z.B. in den Arbeiten von Brumberg (1), Larionov und Brumberg (5) sowie von Buchman und Manojlov (2), Zweifel aufgetaucht sind, ob die bisher von

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sehr zahlreichen Autoren aufgefundenen Absorptionen von Polynucleotiden überhaupt real oder ob sie vielleicht nicht erst durch die UV-Bestrahlung der Zelle induziert worden sind. Zur Klärung solcher und ähnlicher Probleme wäre es wünschenswert, den gesamten technischen Meßvorgang bei der Mikrospektrophotometrie, insbesondere den Vorgang der Registrierung der Meßwerte, ganz erheblich zu verkürzen. Das gelingt beispielsweise durch den Gebrauch eines Kathodenstrahloscillographen.

Die Verwendung von Kathodenstrahloscillographen bei der Photometrie hat also grundsätzlich das Ziel, das Absorptionsdiagramm unmittelbar und praktisch ohne einen ins Gewicht fallenden Zeitverlust auf dem Schirm einer Braunschen Röhre sichtbar zu machen. Es leuchtet ein, daß es auf diese Weise möglich wird, durch die UV-Einstrahlung bewirkte Veränderungen der UV-Absorption, und zwar vor allem auch Veränderungen, die sich nicht gleichmäßig über das gesamte Spektrum verteilen, zur Anschauung zu bringen. Die Konstruktion eines solchen Gerätes stellt an sich kein physikalisches, sondern lediglich ein technologisches Problem dar. Schon vor Jahren wurde auf der ACHEMA ein Gerät gezeigt, mit dem die Absorptionsspektren von Lösungen auf dem Schirm einer Braunschen Röhre sichtbar gemacht wurden. Kürzlich hat auch Nielsen (9) ein Gerät abgebildet, das aus einem Wechsellicht-Monochromator, in dem ein kleiner Spiegel periodisch mit einer Frequenz von ca 1 ... 2 Hz geschwenkt wird, einem Kathodenstrahloscillograph und der photographischen Registriereinrichtung besteht und ebenso eine vollautomatische Aufzeichnung von spektralen Intensitätsverteilungen ermöglicht.

Aus der Tatsache, daß die einzelnen Messungen außerordentlich schnell erfolgen können und die Belastung des Präparates mit einfallendem ultraviolettem Licht sehr gering gehalten werden kann, ergibt sich ohne weiteres die Bedeutung dieses Verfahrens für die Biologie. Praktisch kommt es nun lediglich darauf an, ein Spektralphotometer in geeigneter Weise mit einem Kathodenstrahloscillographen zu verbinden. Die Messung des Präparates kann an sich sowohl mit gleichförmigem Licht erfolgen, d.h. im elektrischen Teil der Anlage mit gleichförmigen Photoströmen, wie auch mit Wechsellicht bezw. mit Wechselströmen. Aus rein äußeren Gründen haben wir vorgezogen, mit Wechselströmen bzw. Wechselspannungen zu arbeiten. Der zur Verfügung stehende Siemens-Einstrahloscillograph vom Typ KE 2072 enthält nämlich einen eingebauten einstufigen Y-Verstärker, der nur bei Wechselspannungen, bei einem Eingangswiderstand von 100 kOhm und einer Eingangsspannung von bis zu 100 V_{eff} im Bereich von 1 Hz ... 35 kHz eine maximale Verstärkung von 200fach ermöglicht. Gleichspannungen lassen

sich demgegenüber nur bis zu 60fach verstärken. Da sich mit einem Kathodenstrahloscillograph ohne weiteres nur Spannungen messen lassen, aber nicht Ströme, ist nun nur noch erforderlich, an einer geeigneten Stelle innerhalb der Verstärkeranordnung des Sekundärelektronenvervielfachers abzugehen und, da es sich um die Verstärkung von Wechselspannungen handelt, über einen Kondensator unmittelbar in den Y-Verstärker des Oscillographen

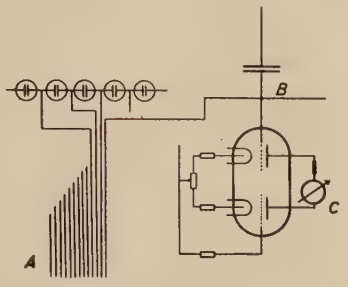

Abb. 1. Prinzipschaltbild der Verbindung zwischen dem Verstärkerteil des Zeiß-Spektralphotometers und einem Kathodenstrahloscillographen. A Multiplier, B Anzeigeinstrument (Mikro-Amperemeter), C Verbindung zum Oscillograph über einen Kondensator von 5 . . . 10 pF Kapazität.

einzugehen. Abb. 1 zeigt den grundsätzlichen Aufbau der elektrischen Schaltung. Man sieht in dieser Abbildung bei A das Kabel, das zum eigentlichen Multiplier führt, und bei C das Mikro-Amperemeter des Anzeigegerätes, das über einen Widerstand mit den Anoden der Röhre in Reihe geschaltet ist. Die bei B über einen Kondensator von 5 ... 10 pF Kapazität abgehende Leitung, die unmittelbar in die Y_{av}-Buchse des Kathodenstrahloscillographen führt, muß abgeschirmt und geerdet verlegt werden. Die auftretende Spannung beträgt etwa 1 Volt.

Neben der Möglichkeit, Wechselspannungen besser verstärken zu können, liegt ein weiterer Vorteil des Wechsellichtverfahrens darin, daß Nebenlicht, das gelegentlich zu Fehlresultaten führen kann, sehr leicht ausgeschieden wird. Bei der normalen Photometrie mit gleichförmigem Licht bzw. mit Gleichspannungen ist das deswegen mitunter schwierig, weil sich oft nicht erkennen lässt, ob ein Ausschlag des Anzeigeinstrumentes tatsächlich nur von der durch Monochromator, Präparat und Mikroskop hindurchgehenden Strahlung herrührt oder vielleicht auch von störenden Lichtbeimischungen, die in irgendwelche Undichtigkeiten des Gerätes eintreten. Bei der hier geschilderten Schaltung werden ausschließlich Wechselspannungen registriert. Sich überlagernde Gleichspannungen, die aus Nebenlicht herrühren, werden nicht aufgenommen. Man braucht z. B. auch nicht mehr den Objekttisch des Mikroskops besonders abzuschirmen oder überhaupt noch im verdunkelten Zimmer zu arbeiten.

Die Intensitäten der aus dem Multiplier hervorgehenden Photoströme

werden als senkrechte Y-Auslenkungen auf dem Schirm des Oscillographen zur Darstellung gebracht. Wechsellicht erhält man auf die bequemste Weise, wenn man vor dem Eintrittsspalt, also zwischen Lichtquelle und Monochromator, eine Scheibe rotieren läßt, die eine hinreichende Anzahl von Sektorenblenden trägt. Wir haben nach zahlreichen Vorversuchen eine 25 cm im Durchmesser große Scheibe benutzt, deren Rand in 72 gleichgroße freie

Abb. 2. Verschiedene Unterbrecherscheiben zur Erzeugung von Wechsellicht mit den zugehörigen Photostromoscillogrammen. Von oben nach unten: 1) Scheibe mit 16,6 % Absorption, zwei Unterbrechungen pro Umdrehung, zeitliche Auflösung des Oscillogramms mit einer Kippfrequenz von 10 Hz. 2) 83,3 % Absorption, zwei Unterbrechungen /U, 10 Hz. 3) 50 % Absorption, 72 Unterbrechungen /U, 100 Hz. 4) Tischventilator mit 12 % Absorption, 3 Unterbrechungen /U, 30 Hz.

und Blendenfelder eingeteilt war, und die mit einem Synchronmotor vom Typ AEG SSLV 375 mit einer Geschwindigkeit von 375 U/min angetrieben wurde. Eine Synchronisierung der Umlauffrequenz ist aber nicht erforderlich. Die Form eines solchen rotierenden Sektors ist nicht beliebig. Erstens soll das Ausmaß der rein mechanisch bewirkten Energieabsorption nicht zu groß sein. Ein Lichtverlust von höchstens 50 % ist gerade noch tragbar. Zweitens hängt die Art der Auslenkung des Oscillogramms von der X-Achse von der Zahl der Sektorblenden bzw. von der Winkelgeschwindigkeit und vom Verhältnis zwischen freien und abblendenden Flächen auf dem Scheibenrand ab. Löst man die resultierenden Photostromimpulse mit Hilfe des Kipp-Gerätes zeitlich auf, so kommt man zu folgenden Gesetzmäßigkeiten, die wir in Abb. 2 an Hand einiger typischer Beispiele übersichtlich dargestellt haben:

Eine Auslenkung von der X-Achse auf dem Oscillographenschirm tritt bei jeder Änderung des Lichtes ein, und zwar in positivem Sinne, d.h. nach oben, wenn Dunkelheit durch Licht, in negativem, wenn Licht durch Dunkelheit unterbrochen wird. Bei niedriger Kippfrequenz entspricht das Verhältnis von Licht und Dunkelheit auf dem rotierenden Sektor genau der Länge von Impuls und Intervall des Oscillogramms in der Zeiteinheit. Bei höheren Kippfrequenzen treten Überlagerungen der Komplexe ein, sodaß

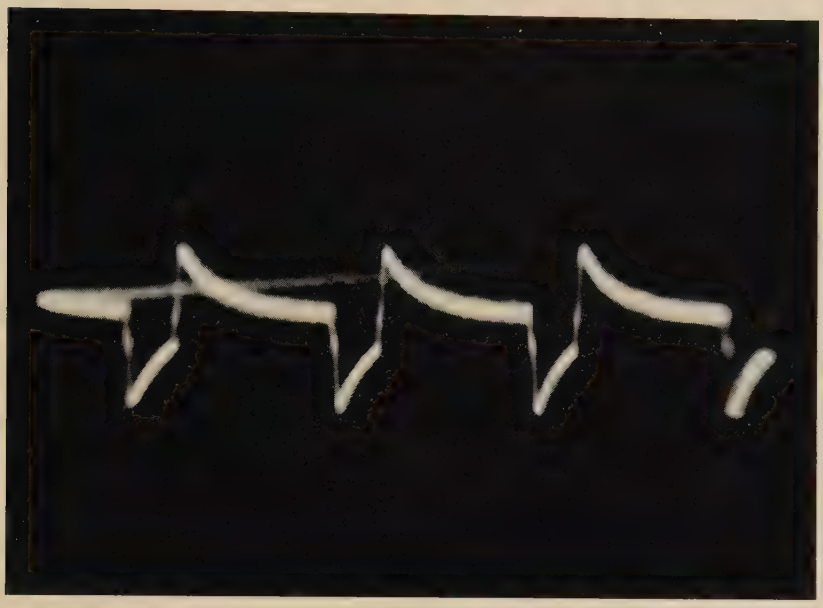

Abb. 3. Oscillogramm eines mit 35 Hz zeitlich aufgelösten Wechsellicht-Photostroms. Ventilator. Contax-Aufnahme.

sich die Intervalle in bestimmten ganzzahligen Vielfachen verkleinern können. Ist der Abstand der Unterbrechungen auf der Scheibe kleiner, als der Zeit entspricht, die für die Auslenkung notwendig ist, so tritt die entgegengesetzte Auslenkung ein, bevor der Kathodenstrahl ein „Intervall" auf der Ruhelinie geschrieben hat. Es leuchtet ein, daß Impulse mit längerdauernden Auslenkungen besser zur Registrierung verwendet werden können als nur kurze Impulszacken. Außerdem ergibt sich aus der Gegenüberstellung der beiden oberen Scheiben mit ihren Oscillogrammen in Abb. 2, daß beide Scheiben bezüglich der Form der Oscillogramme gleichwertig sind, daß aber die zweite Scheibe wegen ihrer hohen Energieabsorption von über 83 % ganz aus der Betrachtung ausscheidet. Die Originalaufnahme eines Oscillogramms eines gut brauchbaren Wechsellicht-Photostroms ist auch in Abb. 3 wiedergegeben.

Ein weiteres Problem besteht darin, daß die zeitliche Ablenkung des Kathodenstrahls meist nicht linear von der Wellenlänge abhängt. Man verbindet am einfachsten einen Schiebewiderstand, an dessen Enden eine Gleichspannung von beispielsweise 110 V angelegt wird, über einen Hebelmechanismus mit der Wellenlängenverstellung des Monochromators. Zufällig

Abb. 4. Selbsttätig registriertes Absorptionsoscillogramm einer wässerigen Lösung von Thionin. Links im Bild UV, rechts Rot. Absorptionsmaximum bei 610 mμ. Contax.

ergab sich bei unseren Untersuchungen, daß die nicht-linearen Abhängigkeiten zwischen Abgriff am Widerstand und Kathodenstrahlablenkung und zwischen Monochromatorverstellung und Wellenlänge sich gegenseitig nahezu aufhoben.

Nach Erfüllung dieser Voraussetzungen ist sowohl die Einstellung der Wellenlänge mit der X-Ablenkung des Oscillographen wie die Intensität des Photostroms mit der Y-Ablenkung gekoppelt. Nun wird zunächst das Oscillogramm der reinen Emissionsstrahlung der Lichtquelle aufgenommen. Diese Kurve ist in Abb. 4 durch die ausgezogene Linie dargestellt. Dann wird die Probe, deren spektrale Absorption gemessen werden soll, in den Strahlengang gebracht und sofort, ohne daß besondere alternierende Leerwertmessungen vorgenommen werden, unmittelbar das Absorptions-Oscillogramm der Probe aufgenommen. Dieser Vorgang kann in Bruchteilen von Sekunden geschehen. In Abb. 4 bezeichnet die punktierte Linie die Absorptionskurve. Naturgemäß ist eine gleiche Genauigkeit der Meßwerte wie bei der gewöhnlichen Mikrospektrophotometrie nicht zu erwarten. Trotzdem gibt die Form des punktiert gezeichneten Absorptions-Oscillogramms, das von einer wässerigen Lösung des Farbstoffs Thionin erhalten wurde, einen Eindruck von der hinreichenden Übereinstimmung mit den Absorptionswerten, die wir (6, 8) auf normalem

Wege von Thionin erhalten hatten. Die besonderen Vorteile dieses Verfahrens, die in der außergewöhnlichen Schnelligkeit der Messung und in der sehr geringen Belastung des Präparates bestehen, wurden oben ausführlich diskutiert.

ZUSAMMENFASSUNG

Durch die Aufzeichnung von Absorptionsdiagrammen mit Hilfe eines Kathodenstrahloscillographen gelingt es, den Vorgang der Absorptionsmessung ganz erheblich zu beschleunigen und damit eine eventuelle Schädigung des Präparates praktisch auszuschalten. Eine Darstellung des elektrischen Schaltprinzips und mehrere auf diese Weise gewonnene Oscillogramme erläutern das praktische Vorgehen.

SUMMARY

The registration of absorption diagrams by using a cathode ray oscilloscope permits a remarkable acceleration of the absorptiometric procedure and avoids any possible alteration of the specimen due to ultraviolet radiation influences. The electrical current principle and some oscillograms obtained in this manner are presented.

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BRIEF NOTES

FINE STRUCTURE OF THE NUCLEAR MEMBRANE IN CELLS FROM THE CHICK EMBRYO: ON THE NATURE OF THE SO-CALLED "PORES" IN THE NUCLEAR MEMBRANE[1]

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Received December 7, 1954

The possibility that the nuclear membrane may be porous was suggested by the work of Callan and Tomlin (5) on amphibian oocyte nuclei. Since this report, nuclear "pores" have been described in a number of forms by various authors, while several other workers have searched and failed to find them (1, 2, 4, 6, 8, 9). The recent report of Gall (7) describes structures in amphibian oocyte nuclei which appear to be piles of small spheres on the cytoplasmic surface of the nuclear membrane. The present report adds several more cell types to the growing roster of those in which circular fine structures have been described on the nuclear membrane. Moreover, it presents an effort to test the validity of the current interpretation that these structures represent true openings in the nuclear membrane.

Experimental. Thin sections were made from portions of 48 hour chick embryos, fixed for 2 hours in buffered 0.25 per cent OsO_4 and embedded in 9:1 N-butyl:methyl methacrylate. In sections in which the nuclear membrane is cut obliquely, circular structures are seen to be associated with it. These structures are 800 Å in total diameter, and show a dense annulus around a lighter center. This dense rim measures 120 Å in average thickness, and does not show further substructure in our micrographs. Annular areas have been observed in several types of embryonic cells: for example in young blood cells, in mesodermal fibroblast cells and in epithelial cells from both ectoderm and endoderm.

Densitometric tracings were made across the structures of interest on positive transparent prints, using the modified Moll microphotometer[2] described by Bennett, Quinton and Mueller (3). These tracings reveal that the annulus is indeed very dense as it appears. But the central "light area" is shown by the tracings to be as dense as, or more often denser than the undifferentiated nuclear membrane outside the annulus. High density, as measured by the tracings, may represent

[1] This work was supported in part by grants from the U. S. Public Health Service (grant number C-1332, C-4); from the Life Insurance Medical Research Fund (grant number G-54-9); and from the Medical and Biological Research Fund of the State of Washington.

[2] We wish to express our appreciation to Professor H. Stanley Bennett, at whose suggestion the densitometric tracings were made.

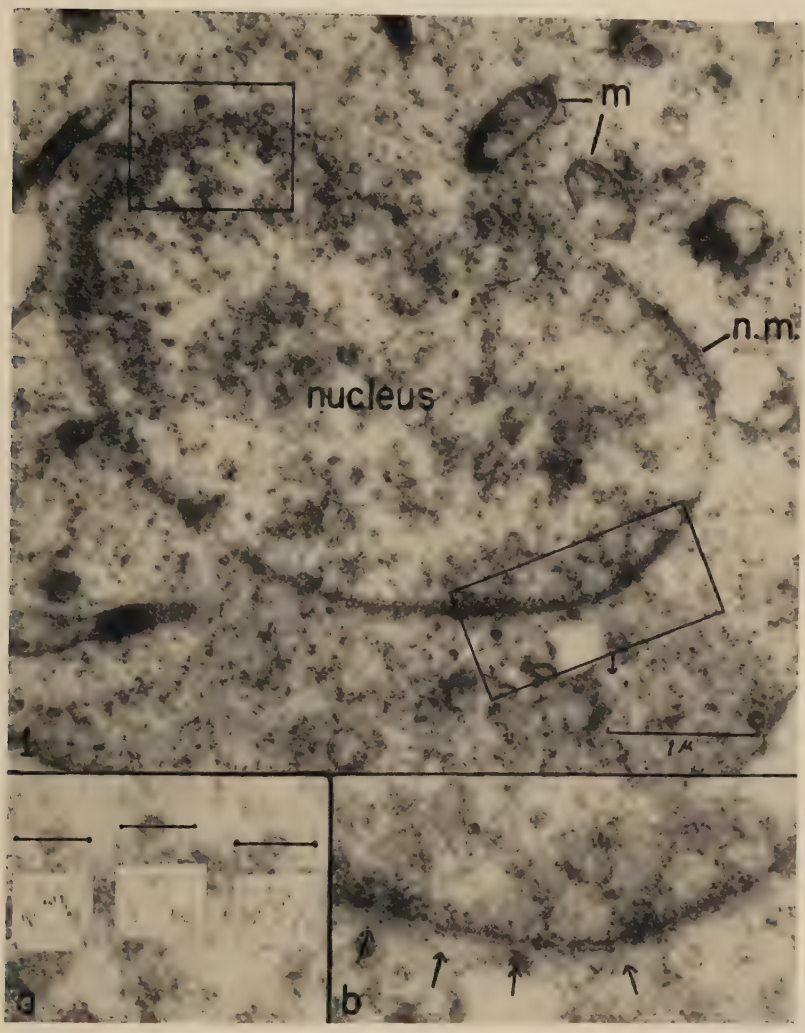

Fig. 1. Nuclear area of a mesodermal cell. Below and on the right the nuclear membrane is cut in cross section and appears double in several places. Above and on the left the nuclear membrane is obliquely cut, showing a number of 800 Å annular areas. N. M., nuclear membrane; M, mitochondria; ×20,000.

Fig. 1 a. Enlarged area from the above nucleus, showing several annular areas. Densitometric tracings were made on positive transparent prints along the pathways shown by black lines. The scale of the tracings has been reduced so as to correspond with the same area in the print. Higher densities are recorded as upward deflections of the curve. The tracings cast doubt on the interpretation that the structures represent simple openings in the nuclear membrane. In every case, the central area is at least as dense, if not more dense, than the surrounding nuclear membrane. True openings would be less dense and produce a tracing going below that of the surrounding undifferentiated membrane. It is therefore suggested that the structures in question, if they are pores, are more complex than previously supposed. ×40,000.

Fig. 1 b. Enlargement of a cross sectioned portion of the nuclear membrane. The double structure appears to be interrupted by occasional gaps or irregularities, which may correspond to cross sections of the annular areas. ×40,000.

innate density of the tissue, or it may reflect increased density due to osmium binding capacity of certain areas. True openings in the nuclear membrane would not be expected either to bind osmium, or to be intrinsically more dense than the surrounding membrane. The revelation that the apparently "light area" within the dense annulus is in reality more dense than the surrounding membrane suggests that, in this tissue at least, the structures in question are either not pores at all, or are pores whose apertures are filled with some osmophilic substance.

When cut in cross section, the nuclear membrane appears in favorable sections to be double, the constituent membranes together measuring approximately 300 Å in thickness. Many of these areas have been carefully searched for some evidence of the above structures. Occasional gaps or irregularities can be seen, but at present it is not certain whether these correspond to cross sections of the dense rimmed areas seen in obliquely cut membranes.

Summary. Nuclei of various types of cells from the chick embryo show dense-rimmed circular areas, 800 Å in diameter, closely associated with the nuclear membrane. These structures correspond to the "pores" described in the nuclear membranes of various forms by other authors. Densitometric tracings have been made across these annular areas in order to test the interpretation that they represent pores. The central "light area" within the annulus is shown to be more dense, rather than less dense, than the surrounding nuclear membrane. Thus the annular structures seen in obliquely cut nuclear membranes do not seem to represent simple openings, but are apparently more complex than previously supposed.

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THE FERTILIZATION REACTION IN ISOLATED CORTICAL MATERIAL FROM SEA URCHIN EGGS

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Received December 7, 1954

Fertilization is initially an interaction between the egg and sperm surfaces. The ability of the egg to exhibit a normal cortical reaction (membrane elevation, cortical granule breakdown, hyaline layer growth and the prevention of polyspermy) develops gradually during and immediately after the maturation divisions. During meiosis, the cortical granules begin to migrate toward the cell periphery (4, 5); if fertilization occurs before this process is complete, faulty membrane elevation occurs and polyspermy is frequent. If, after the cortex has fully differentiated, this region of the cell is damaged or removed as in an exovate, a typical cortical reaction fails to occur and no block to polyspermy is established (7).

Because of the importance of the cortex not only in the cortical reaction at fertilization, but also in the early events leading to cleavage and differentiation (3), it is advantageous to develop many techniques with which to study this region of the cell. It is well known that eggs from which the jelly layer has been removed with acid sea water become adhesive to glass surfaces. Such eggs often adhere to the bottoms of containers and burst when an effort is made to dislodge them. A fortuitous observation showed that sometimes an egg could be pulled away from a glass surface in such a way that a single or double layer of cortical material was left attached to the glass.

An experiment was designed to determine whether this isolated cortical material could be obtained reproducibly and whether it could be used for physiological experimentation. The eggs of either *Psammechinus* (*miliaris* on the Swedish west coast, *microtuberculatus* at Naples) or *Sphaerechinus granularis* (Naples) were transferred from a thick suspension into a large volume of sea water at pH 4.5 and stirred rapidly. After 20–30 seconds the suspension was flooded with slightly alkaline sea water (pH 8.5). Such brief acid treatment usually removed most or all of the egg jelly and rendered some of the egg surfaces adhesive to glass.

Two ordinary cover glasses (soft-glass) were separated by four beads of melted sealing wax at the corners and allowed to cool. A thick suspension of jelly-free eggs in sea water was pipetted into the space between the cover glasses (less than 1 mm). After five minutes the cover glasses were inverted and any eggs not adhering to the

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glass were removed by a stream of water from a pipette on one side of the cover glasses to a blotter on the other. Under ideal conditions, a large number of eggs then hung to the lower surface of the upper cover glass. Some of these adhering eggs were pulled away by means of a capillary pipette, leaving others to serve as a control. When the glass surface to which the eggs had been attached was examined with an oil immersion objective, it was noted that fragments of cortical material in single and double layers had been left behind. Some double layers of cortex surrounded a small amount of endoplasmic material; others did not. The cortical material remaining when the egg was torn away compared identically in visible structure to the cortex of the control intact eggs still adhering to the glass, except that the edges of the cortical patches were often lined with fibrillar projections extending for a few microns along the glass.

Strips of single layer, isolated cortical material from 10 to 25 microns long were brought into critical focus during insemination. When sperm were introduced between the cover glasses, a normal fertilization reaction took place in the control eggs; sperm also attached in moderate numbers to the single and double layer patches of isolated cortex and caused complete breakdown of the cortical granules. Within seconds, a very thin fertilization membrane became visible surrounding the edges of the patches. The details of the cortical response apparently did not differ from the normal fertilization reaction in the cortex of intact eggs, except that hyaline layer thickness could not be ascertained. Cortical granule breakdown was independent of the presence of any endoplasmic material; this observation casts doubt on any conduction role attributed to the endoplasm in the cortical reaction.

Attachment to this particular kind of glass apparently did not seriously influence the cortical reaction. Intact eggs attached to the upper cover glass showed complete cortical granule breakdown. This result is in contrast to, but not in contradiction with the results obtained by Runnström and Kriszat (7), who found evidence to indicate that attachment to one kind of glass prevented the propagation of the fertilization impulse. It is clear that different kinds of glass affect the cortical reaction differently (2, 7).

In one experiment, parallel cortex isolations were carried out on eggs of *Psammechinus microtuberculatus* and *Sphaerechinus granularis*. An attempt made to perform cross-fertilization failed in both directions. Subsequent addition of homologous sperm elicited a typical cortical reaction.

Some eggs which had lost part of their cortical material were examined. Most of these exhibited cortical tears under which a smooth protoplasmic surface was exposed; some, however, showed injury typically observed when the surface of an egg bursts and seals again rapidly. Where cortical tears were present without any sign of further external injury, there was no sign of incipient cytolysis underlying the bare cytoplasmic surface. This observation suggested the possibility that whatever structure functions as the plasma membrane might lie below the cortex. Parpart and Laris (6) have recently concluded from permeability experiments that the plasma membrane must, in fact, lie beneath the cortex.

The fact that cortical material can be torn from the cell surface and still maintain a high degree of its structural and functional integrity indicates either an ability to form a precipitation membrane with phenomenal speed, or else that the cortex is surrounded by membranes on both sides or needs no membranes at all.

The latter membraneless state was suggested by the manner in which the cortical granules were expelled into the medium without any apparent membrane resistance in eggs the vitelline membranes of which had been digested away by trypsin ((8) and personal observation). Furthermore, chemical changes occurring in the cortex appear to be highly susceptible to environmental conditions (pH, cation changes etc.), indicating a highly permeable character for this region of the cell (1).

The isolation of cortical material has provided another technique for the study of the cell cortex. The method is tedious because different lots of eggs vary in their susceptibilities to jelly removal by acid sea water, but the study of the effect of various activating agents, enzymes and other substances on the cortex can now be pursued without the complications which normally arise in the indirect effects on the cortex by way of injury to the endoplasm.

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THE PROTECTIVE PROPERTY OF THIOUREA AGAINST RADIATION-INDUCED CHROMOSOME ABERRATIONS[1]

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Received December 12, 1954

Several authors have reported on the modifying effect of anoxia on the production of radiation induced chromosome aberrations (1, 5, 6, 7, 8, 20, 22). Giles and Riley (6, 7) demonstrated that oxygen was involved in an indirect radiation mechanism and had no influence on the recovery process in *Tradescantia*. Schwartz (20) claims, however, that broken chromosomes are more capable of rejoining in anoxia than in the presence of oxygen.

Evidence of an indirect radiation effect is also demonstrated by chemical means. Mikaelsen (12, 13, 14, 15) has shown that in the presence of certain chemicals such as glutathione, cysteine, sodium hyposulphite and sodium cyanide the frequencies of chromosome fragments were markedly reduced during exposures to small doses of chronic γ-irradiation.

Similar evidence has also been presented by Riley (19) and Wolff (25) with Sodium hyposulphite and BAL and by Forssberg and Nybom (4) with cysteine during acute exposures to X-rays. Mikaelsen (13, 15) has also stated that thiourea is a protective agent against radiation induced chromosome aberrations, and the results of the experiments will be presented here.

Material and methods. Cuttings of *Tradescantia paludosa* (Sparrows clone B 2–2) were used as experimental material. The method used to initiate root development is described earlier (12, 14). When the cuttings showed good development of primary roots, they were transferred to specially designed lucite vessels which contained Hoagland and Snyder's (9) nutrient solution. The vessels were $30 \times 12 \times 2$ cm in size. The side of the vessel facing the source was 3 mm thick. After 24 hours in the nutrient solution, the cuttings were transferred to fresh nutrient solutions containing various concentrations of thiourea. After one hours preabsorption, the plants, still in the solutions of thiourea, were exposed to continuous γ-irradiation from 8.5 Curie Co^{60} source for 48 hours. The source was located in a cylindrical, stainless steel container with a wall thickness of 0.8 mm on the front side. The side walls were 1 mm thick. The source holder was placed on a lead cylinder inside a cylindrical aluminium pipe with wall thickness 1 mm. The pipe was located near

[1] This paper is published as Report no. 6 from the Institute of Genetics and Plant Breeding (Director: Prof. dr. H. Wexelsen), Vollebekk, Norway, where the analyses are carried out. The experiments are performed at Brookhaven National Laboratory, Biol. Dept., Upton, New York, U.S.A. under the auspices of the U.S. Atomic Energy Commission. Acknowledgement is made to Dr. A. H. Sparrow of this laboratory. The study is supported with grants from the Norwegian Research Council for Science and the Humanities.

TABLE I

The effect of Thiourea on frequencies of chromosome aberrations after chronic γ-irradiation of a dose rate of 25 r/day for 48 hours.

Conc. of Thiourea	No. of cells	No. of fragments	No. of fragments per 100 cells	Per cent protection	No. of bridges	No. of bridges per 100 cells
None	439	84	19.1	0	7	1.6
5×10^{-5} M	578	103	17.8	7	13	2.3
10^{-4} M	680	73	10.7	44	21	3.1
10^{-3} M	638	63	9.9	48	11	1.7

the corner of an L-shaped lead and concrete shield, which gave an irradiation field of about 90°. Dosages, as measured by the film badge method (2) indicated that the plants received approximately 25 r/day and hence a total dose of 50 r.

Immediately following the end of the 48 hour exposure period the root tips were fixed in alcohol-glacial acetic acid (3:1). Slides were prepared using the acetocarmine squash technique. The frequencies of both acentric fragments and bridges were studied in anaphase of root tip cells. Conclusions of the effects of irradiation and thiourea treatments, however, were derived from the fragment frequencies only, since bridges occurred very infrequently.

Results. As demonstrated earlier (12, 14, 15) the yield of acentric fragments following exposures to chronic γ-irradiation of 25 r/day was approximately 20 fragments per 100 anaphase cells in the root tips of *Tradescantia paludosa*. In the thiourea experiment the untreated control series yielded 19.1 fragments per 100 cells (Table I). At a concentration of 5×10^{-5} M a slight and insignificant decrease in fragment frequency (17.8 per 100 cells) of 7 per cent was obtained (Table I).

At a twice as strong concentration of 10^{-4} M a marked and significant reduction of 44 per cent in fragment frequency was attained. The maximum possible decrease was apparently reached around this value, since at 10^{-3} M, a tenfold increase in concentration, there is 9.9 fragments per 100 cells, a frequency that is only slightly less and insignificantly different from that of the second highest concentration used. Thus, the maximum effect obtained with thiourea gave a significant reduction in fragment frequency of 48 per cent of that found in irradiated controls. The effectiveness of thiourea increased with concentrations up to 10^{-3} M.

Reunions resulting in bridge formation at anaphase were infrequent (Table I), yielding only 1.6 bridge per 100 anaphase cells. The bridge frequencies for the thiourea treated series were 2.3, 3.1 and 1.7 per 100 cells respectively at 5×10^{-5} M, 10^{-4} M and 10^{-3} M. There seems to be no significant differences between the treated and untreated series. No conclusions, however, regarding the effect of thiourea on restitution can be reached on the basis of these data, since the frequency of bridges is so low.

Discussion. Among others Patt (17, 18) and Sparrow and Forro (21) have reviewed and discussed protective mechanisms and different factors influencing the radio-

sensitivity of cells. Although an indirect effect of radiation on chromosomes is demonstrated by several investigators, it still remains to be answered how these indirect mechanisms operate. Thoday and Read (23) and Giles (7) have suggested the importance of H_2O_2 in the production of chromosome aberrations. Mikaelsen (12, 13, 14, 15) has emphasized and discussed possible interpretations by reactions of inorganic or organic free radicals and peroxides and metabolic alterations.

The present demonstration of the protective property of thiourea against radiation induced chromosome breakage, reveals no evidence of the mechanism by which thiourea exerts its modifying effect. The experimental results should still be of some interest.

The chemical formula of thiourea is usually presented as $NH_2—S—NH_2$. In oxidation processes, however, it reacts as a sulphydryl compound $NH_2—SH=NH$ (24). In previous experiments (12, 13, 14, 15) the sulphydryl compounds glutathione and cysteine gave a reduction in fragment frequency of 50–60 per cent. In the presence of thiourea the fragment frequency is reduced by 48 per cent, which is almost the same as was obtained with the other sulphydryl compounds. Since these chemicals are so similar in effect, it may be concluded that the –SH groups are mainly responsible for their protective property, as is usually assumed.

Thiourea has also proved to be effective in reducing the lethal effect of whole body X-irradiation in mice (16). The reduction in mortality caused by thiourea is only equivalent to a reduction in the effectiveness of radiation by 10 or 15 per cent. In Forssberg's (3) X-ray experiments on growth of *Propionibacterium pentosaceum*, thiourea gave 22 per cent while cysteine yielded 45 per cent protection. Thus, thiourea proves to be less effective as protective agent in these experiments than in the present experiments on chromosome breakage in *Tradescantia paludosa*.

The processes by which protective agents exert their effects are certainly complicated and complex and no clear evidence has been presented so far regarding the true nature of those processes, which may be quite different in different organisms and biological systems. The discussion of the effect of CO on radiosensitivity of chromosomes by King *et al.* (10, 11) demonstrates clearly the difficulties in interpretation of radiation effects inside such complicated systems as cells and cell nuclei.

Summary. The protective property of thiourea against chromosome fragmentation induced by chronic γ-radiation is demonstrated. Its effectiveness increased with concentrations between 5×10^{-5} M and 10^{-3} M. At 10^{-3} M maximum reduction in fragment frequency by 48 per cent was obtained.

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STUDIES ON THE EFFECT OF OXYGEN ON CHROMOSOME BREAKAGE INDUCED BY 8-ETHOXYCAFFEINE

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Received February 1, 1955

Previous experiments have shown that the frequency of chromosome aberrations produced in *Allium* by 8-ethoxycaffeine (EOC) is dependent on the oxygen concentration during the treatment (4, 5). These experiments have now been repeated with root tips of *Vicia faba* as experimental material.

The technique was the same as in the *Allium* experiments, except that the standard treatment with 10^{-2} M EOC was prolonged to 1 hr. 45 min. As a rule no pre- or post-treatments of root tips with oxygen or nitrogen were performed. In order to facilitate the analysis of the aberrations, the roots were treated the last 3½ hours of the recovery period in 0.05 per cent colchicine. Most of the cytological observations were confined to chromatid aberrations obtained 5½ hours after the EOC treatment. In contrast to what was previously found in *Allium*, chromatid interchanges predominated among these aberrations.

The aberrations studied 5½ hours after the treatment were induced in late interphase or possibly in early prophase. In some experiments aberrations induced in earlier stages of interphase were studied 19 hours after the EOC treatment. The main aberrations obtained after a recovery period of 19 hours were isolocus breaks, the majority of which were SU (terminology according to Thoday (10)). In agreement with previous observations (6, 9) a considerable part (50–80 per cent) of the isolocus breaks were localized in the nucleolar constriction (NC). As a rule the lower the dose, the greater the percentage of isolocus breaks of NC type.

The results of EOC treatments of *Vicia* root tips in oxygen and nitrogen atmosphere confirmed those previously obtained in *Allium*. As in the *Allium* experiments, chromosomes treated in oxygen showed a pronounced stickiness 1 hour after the treatment, whereas practically no stickiness was observed when the treatment was performed in nitrogen. EOC treatments in oxygen resulted in a high frequency of chromatid interchanges 5½ hours after the treatment. In nitrogen atmosphere the number of exchanges per 100 cells was less than 1/30 of that in oxygen (Table I). In some experiments, fixations were made with short intervals from 1 to 24 hours after the treatment. The effect produced in oxygen was always several times larger than that in nitrogen, which shows that the difference in aberrations frequency found 5½ hours after the treatment is not due to a shift of the aberration maximum as a result of a different speed of mitosis after the treatments.

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TABLE I

The effect of oxygen tension and of various chemicals on the frequency of structural chromosome changes produced by 10^{-2} M 8-ethoxycaffeine.

Period of treatment hours	Recovery period (at 20 °C) hours	Treatment temperature °C	Treatment atmosphere	Chemical added		Number of metaphases analysed	Abnormal metaphases per cent	Aberrations per 100 cells			
				formula, abbreviation	concentration M			Chromatid breaks	Isolocus breaks	Exchanges	Minutes
1 ¾	5 ½	20	nitrogen	—	—	400	6	1.8	1.8	3	0
1 ¾	5 ½	20	air	—	—	400	64	14.5	19	85	0
1 ¾	5 ½	20	oxygen	—	—	200	73.5	24.5	16	95.5	0
1 ¾	5 ½	20	nitrogen	H_2O_2	5×10^{-3}	200	44	8	13.5	74	0
1 ¾	5 ½	20	air	NaN_3	4×10^{-4}	100	3	2	0	1	0
2	19	9	nitrogen	—	—	100	2	0	1	1	0
2	19	9	air	—	—	100	32	1	34	5	1
2	19	9	air	DNP	10^{-4}	150	4	0	2	0	2
4	19	9	nitrogen	—	—	100	6	1	4	1	2
4	19	9	air	—	—	100	64	4	67	11	2
4	19	9	air	DNP	10^{-4}	200	8.5	0	9	0	0
8	19	9	nitrogen	—	—	300	14.3	2.7	8	6	0
8	19	9	air	—	—	100	93	3	152	60	0

In addition to these observations, a number of new facts were obtained. Thus it was found that only the oxygen concentration during the actual EOC treatment is effective in modifying the frequency of aberrations produced. Pre- or post-treatments in nitrogen or oxygen had no effect.

It was further shown by the experiments that there is very little difference between the frequencies of aberrations obtained in air (21 per cent oxygen) on the one hand and in oxygen on the other (Table I). In most of the subsequent experiments, therefore, air instead of oxygen was bubbled through the treatment solution.

So far there is a striking similarity between the effect of oxygen on the production of chromosome aberrations by EOC on the one hand and by X-rays on the other. (A detailed review of the oxygen effect in the case of X-rays has recently been published by Giles (2).) A difference exists, however, in regard to the magnitude of the effect in nitrogen. In the case of X-rays, the effect in nitrogen is about $\frac{1}{3}$ of that in air or oxygen. When the effect of EOC was scored $5\frac{1}{2}$ hours after the treatment, it was found that about 30 times more chromatid interchanges were produced in oxygen than in nitrogen. Similar rations were obtained when isolocus breaks were scored 19 hours after the treatment (Table I). On the whole, even with the highest doses employed, very little breakage is obtained with EOC in the absence of oxygen.

The explanation of the oxygen effect, given in the case of X-rays, can hardly be valid in the case of EOC, since it is difficult to imagine how EOC should be able directly to produce free radicals in water. Possible explanations in the case of EOC seemed to be: (i) that EOC is inactive as such, but that in the presence of molecular oxygen it is converted *in vivo* to an active compound; (ii) that molecular oxygen directly or indirectly influences the state of the chromosomes in such a way that structural chromosome changes are produced in the presence of EOC.

The first of these alternatives seemed unlikely already when it was found that the radiomimetic effect of 1,3,7,9-tetramethyluric acid (TMU) is influenced in the same way as that of EOC by the concentration of oxygen during the treatment. The two purines differ considerably as to their chemical structure and reactions, and their degradation products should probably be entirely different. Furthermore, TMU is a very stable compound, which is not likely to be oxidized or changed *in vivo*. Therefore, when later considerable evidence accumulated in favour of the second alternative, the first was rejected at least for the present.

It was found that if root tips of *Vicia* were treated aerobically with a solution containing 10^{-2} M EOC, as mentioned above, but in addition potassium cyanide (KCN) or, rather, sodium azide (NaN_3) in low concentrations (10^{-4}–10^{-3} M), the stickiness effect produced, as well as the frequency of chromosome aberrations obtained various periods after the treatment, were of the same magnitude as those obtained after treatments in nitrogen (Table I). KCN and NaN_3 are chemically very different, but they have at least one property in common: they both inhibit a number of iron-containing enzymes, of which cytochrome oxidase is the most important in this connection. This enzyme catalyzes the oxidation of cytochrome C by molecular oxygen, and if it is inhibited, the other members of the respiratory chain are stabilized in the reduced form (as under anaerobic conditions) and respiration ceases. The effect of KCN and NaN_3 on root respiration has been studied by Marsh and Goddard (8).

The fact that the radiomimetic effect of EOC is influenced by inhibitors of cy-

tochrome oxidase shows that the oxygen effect in the case of EOC is operating by the way of the respiratory chain and that molecular oxygen has no effect if the respiratory chain does not function.

In parallel experiments with X-rays, NaN_3 had no effect on the frequency of chromosome aberrations produced, which indicated that the explanation of the oxygen effect in this case probably is another.

In experiments with EOC it was further found that if the treatments were performed in the absence of oxygen, but with 5×10^{-3} M hydrogen peroxide (H_2O_2) in the treatment solution, an aberration frequency of the same magnitude as that produced under aerobic conditions was obtained. The explanation of this could either be that molecular oxygen is produced *in vivo* from H_2O_2 as a result of catalase action or, what is more probable, that an oxidation of cytochromes by H_2O_2 is catalyzed by peroxidases. That plant peroxidases are able to catalyze the oxidation of cytochrome C by H_2O_2 has been shown by Chance (1).

In these experiments H_2O_2 alone did not produce any chromosome aberrations in *Vicia*.

The fact that the oxygen effect operates by the way of the respiratory chain could mean that electrons are transferred by the cytochromes to molecular oxygen from some compound, which has to be in oxidized form if EOC shall be able to induce chromosome breakage. But the oxygen effect could also have something to do with the fact that respiration is coupled to phosphorylation and the synthesis of energy-rich phosphate bonds, the importance of which is that they constitute "the chief mechanism for transferring energy to totally unrelated synthetic processes" (3).

Since certain compounds, e.g. dinitrophenol (DNP), have been found to uncouple phosphorylation from oxidation (7), there seemed to be a clear possibility to test the validity of the second of these alternatives. Therefore, in a number of aerobic experiments, DNP was added to the EOC solutions. The effect was scored after a recovery period of 19 hours. As appears from Table I, EOC produced an effect in the presence of 10^{-4} M DNP of the same magnitude as in the absence of oxygen. It may be added, that when DNP was substituted in the treatment solution by the same concentration of *o*-nitrophenol, which is much less active as an uncoupling agent, no reduction in the frequency of aberrations occurred.

The experiments indicate that compounds containing energy-rich phosphate bonds are involved in the production of chromosome aberrations by EOC, and that oxygen is of significance for the radiomimetic effect because in its absence the formation of energy-rich phosphate is strongly reduced.

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CYTOCHEMISCHER NACHWEIS DER NUCLEOPROTEIDE DURCH ENZYMATISCHEN ABBAU

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Eingegangen am 2. Februar 1955

Wie von Kaufmann u. a. (2, 4) gezeigt wurde, kann durch schrittweise Behandlung fixierter Präparate höherer Zellen mit nucleinsäurespaltenden Enzymen und Pepsin die Affinität der jeweiligen Zellelemente für saure Farbstoffe aufgehoben werden. Nach Ansicht dieser Autoren führt eine Kombination von Ribonuclease (R.ase) und Pepsin — unabhängig von der Reihenfolge (3, 1) — zu einem Abbau der Ribonucleoproteide (entsprechendes wird für Desoxyribonuclease (D.ase) und Pepsin nicht ausdrücklich behauptet). Nach Einwirkung von Nucleasen bzw. Pepsin allein soll die lichtoptisch sichtbare Zellstruktur wegen des Verbleibens des Protein- bzw. des Nucleinsäureanteils intakt bleiben.

Frühere Untersuchungen an gramnegativen Bakterien (10, 11) hatten ergeben, dass die Nucleoproteide sich durch Behandlung mit einer oder beiden Nucleasen und nachfolgende Pepsineinwirkung spezifisch abbauen lassen. Dabei konnte aus elektronen- und phasenkontrastmikroskopischen Befunden, die durchweg übereinstimmten, die Substanzverminderung weit sicherer beurteilt werden, als dies aus gefärbten Präparaten möglich ist. Die umgekehrte Reihenfolge (erst Pepsin, dann Nucleasen) führte nicht zum Abbau der Nucleoproteide. Demnach dürfte zumindest der überwiegende Teil der Proteine dem Pepsinangriff erst *nach* Abbau der Nucleinsäuren zugänglich werden, und zwar im Rahmen der durch die Nucleasebehandlung vorgegebenen Spezifität.

Die Anwendbarkeit dieser Vorstellungen auf cytologisches Material wurde an Carnoy- oder Alkohol-Eisessig (3 : 1)-fixierten Paraffinschnitten von Chorioallantoismembran des Hühnerembryos und Mäusehaut unter Benutzung kristallisierter Enzyme (Worthington) entsprechend den früher (10, 11) geschilderten Bedingungen geprüft. Die Schnitte wurden nach Giemsafärbung sowie insbesondere ungefärbt im Phasenkontrastmikroskop unter besonderer Berücksichtigung der epithelialen Zellschichten untersucht. Während die Nucleasen den erwarteten Effekt auf die Färbbarkeit nach Giemsa hatten, veränderte sich der Phasenkontrast ungefärbter Schnitte nach Einwirkung einer oder beider Nucleasen nicht. Auch Pepsin allein führte nicht zu einer Strukturänderung (Abb. 1). Behandlung mit R.ase und Pepsin hatte dagegen eine völlige Aufhellung des Cytoplasmas und eine weitgehende der Nucleolen zur Folge (Abb. 2), während das Chromatin der Zellkerne relativ kontrastreich hervortrat. Umgekehrt erbrachte D.ase und Pepsin eine Aufhellung lediglich des Chromatins. Nach Anwendung von R.ase, D.ase und Pepsin waren nur noch die Umrisse von Zellen und Kernen zu erkennen (Abb. 3). Die Kerne der Hühnererythrozyten wurden allerdings nicht in allen Fällen völlig abgebaut. Als Kontrollversuch zur

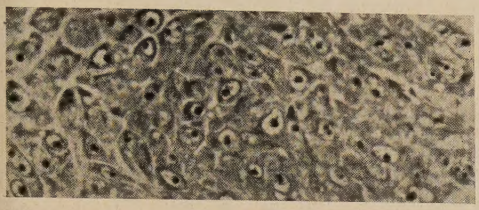

Abb. 1: Chorioallantois des Hühnerembryos, Ektoderm. Dest. Wasser (pH 6, 2 Std.), Pepsin (pH 2, 0,02%, 2 Std.) (wie unbehandelt). Phasenkontrastmikroskop. 500 : 1.

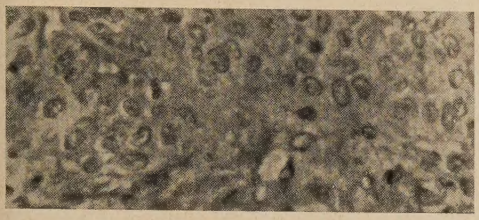

Abb. 2: Behandlung mit R.ase (pH 6, 0,05%, 1 Std.) und Pepsin (0,02%, 2 Std.).

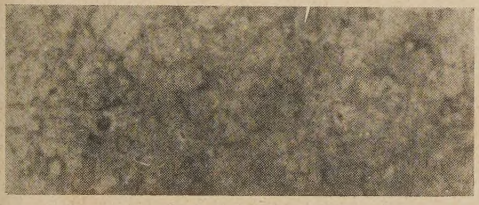

Abb. 3: Behandlung mit R.ase (0,05%, 1 Std.), D.ase (pH 6, 0,05%, 2 Std.) und Pepsin (0,02%, 2 Std.).

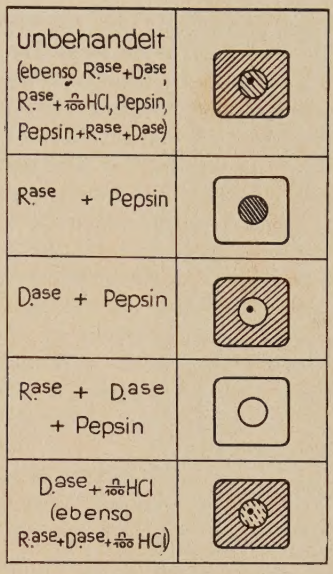

Abb. 4: Enzymatischer Abbau epithelialer Zellen nach Alkohol-Eisessig-Fixierung (Phasenkontrastmikroskop).

Pepsinbehandlung zeigte $n/100$ HCl im Anschluss an R.ase angewendet keinen Effekt. Dagegen erfolgte nach D.ase und $n/100$ HCl eine partielle Aufhellung des Chromatins (Abb. 4). Bemerkenswert war weiter, dass eine oder beide Nucleasen im Anschluss an Pepsin angewendet nicht zu einer sicheren Kontraständerung führten. Es handelt sich also nicht um einen Summationseffekt mehrerer Abbauvorgänge, die für sich unterhalb der Grenze der Nachweisbarkeit liegen.

Da das Phasenkontrastmikroskop allein keine eindeutigen Aussagen über die Massenverhältnisse zulässt, wurde die Deutung der Befunde durch eine elektronenoptische Kontrolle gesichert. Hierfür eigneten sich Pseudoabdrucke von anämischem Rattenblut (8) nach Fixierung mit Alkohol-Eisessig. In diesen Präparaten hafteten stets die Kerne der Leukozyten und ein Teil der basophilen Substanz der Reticulozyten am Film. Während die Nucleasen allein ohne Wirkung auf den Phasen- und elektronenoptischen Kontrast blieben, hatte Pepsin eine Schrumpfung der Leukozytenkerne zur Folge. Die Behandlung mit D.ase und Pepsin führte zu vollständigem, mit D.ase und $n/100$ HCl zu partiellem Abbau der Leukozytenkerne. R.ase und

Pepsin entfernte die basophile Substanz der Erythrozyten, liess jedoch die Leukozytenkerne unbeeinflusst. Die Anwendung der Nucleasen nach Pepsin hatte wiederum keinen entsprechenden Effekt. Bezüglich der Leukozytenkerne stimmten Phasen- und elektronenoptische Befunde vollkommen überein; dabei verhielten sich die verschiedenen Leukozytentypen — im Widerspruch zu den Befunden von Laves (6) — durchaus einheitlich. Dagegen war eine Beurteilung der Reticulozytensubstanz nur elektronenoptisch mit genügender Sicherheit möglich.

Über das Vorhandensein von Ribonucleinsäure im Kern liess sich mit dieser Methode nichts aussagen, da sich mit ihr nur die massenmässig überwiegenden Komponenten erfassen lassen. Die Wirkung von $n/100$ HCl nach D.ase ist wahrscheinlich durch Herauslösung des im Chromatin vorliegenden Histonanteiles zu deuten — bekanntlich sind Histone in verdünnten Säuren löslich (5, 7) —, während das an Desoxyribonucleinsäure gebundene Nicht-Histon-Protein nach D.ase-Behandlung nur durch Pepsin abgebaut werden kann. Dieser Einfluss verdünnter HCl im Anschluss an die Nucleasen angewendet steht in Parallele zur Veränderung der Lichtgrünfärbung (2, 4).

Die hier an höheren Zellen erhaltenen Ergebnisse stützen demnach unsere an Bakterien gewonnenen Anschauungen in vollem Umfang. Auch bei den desoxyribonucleinsäure-haltigen Innenkörpern von Viren der Pockengruppe konnte ein spezifischer Abbau mit D.ase und Pepsin — und zwar nur in dieser Reihenfolge — erzielt werden (9). Ebenso verhielten sich die Blutparasiten *Haemobartonella muris* und *Eperythrozoon coccoides* im Prinzip ähnlich (12). Ein spezifischer Abbau der Nucleoproteide durch die jeweilige Nuclease und nachfolgende Behandlung mit Pepsin scheint demnach bei mikrobiologischen und cytologischen Objekten gleichermassen möglich zu sein.

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